Glutenin Polymers:
The In Vitro to In Vivo Transition

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Both hybrid and unmodified glutenin protein subunits expressed in bacteria and transgenic wheats through the use of genetic engineering techniques are providing exciting new information about how glutenin subunits combine to form glutenin polymers. Mixograph testing of doughs from transgenic lines has provided clues as to how the structure and disulfide cross-linking of the glutenin subunits contribute to variation in mixing character among wheat cultivars. Computer molecular models are also contributing to a better understanding of how the structures of glutenin subunits determine properties of glutenin polymers. In this interpretive review, I combine information from the expression of recombinant proteins in vivo—in bacteria or in transgenic wheat lines—with pertinent in vitro results from the earlier literature to construct a model that attempts to explain the relative importance of various glutenin subunits to dough mixing strength and elasticity.

N-Terminal Domains of Y-Type HMW-GS Form Predominantly a Single Intermolecular Disulfide Bond

High molecular weight glutenin subunits (HMW-GS) have a number of allelic forms, which differ among cultivars. The various combinations of these subunits found in wheat cultivars (usually four or five are expressed in any given cultivar) show significant correlations with variations in quality (1,2), especially for hexaploid bread wheats. Structurally, the HMW-GS have three domains, small N-terminal and C-terminal domains of about 100 and 50 amino acids, respectively, and a large central domain made up of repeating sequences. All the cysteine residues that form intra- and intermolecular disulfide bonds are situated in or close to the N- and C-terminal domains. The repeating sequence domain is rich in glutamine residues that are capable through their side chains of strong hydrogen-bonding inter-

actions with other gluten proteins. Hydrogen bonding contributes strongly to the cohesiveness of gluten proteins in a dough (3).

Blechl and Anderson (4) combined the DNA coding for the N-terminal domain of HMW-GS Dy10 with the DNA coding for the repeating sequence and C-terminal domains of HMW-GS Dx5 to form a Dy10/Dx5 hybrid gene. They introduced the constructed hybrid gene into wheat to obtain transformed lines in which the hybrid protein was overexpressed. Shimoni and co-workers (5) showed that in lines that overexpress the Dy10/Dx5 hybrid gene a substantial part of the hybrid protein is present in the mature grain as a monomer. This is surprising in that so far no monomeric forms of HMW-GS have been found in normal wheat cultivars; HMW-GS are apparently always incorporated into the glutenin polymer chains. Furthermore, Shimoni and co-workers (5) showed that the monomeric form is predominantly circular in nature, in the sense of having a disulfide bond that links the single cysteine at the C-terminal end of the molecule with one of the five cysteines found in the N-terminal domain of the hybrid (Fig. 1).

Tao and co-workers (6) found that proteolytic digestion of glutenin with endoproteinase Lys-C yields peptides that link the single cysteine of an x-type subunit with one of the five cysteines located at the N-terminal end of a y-type subunit (Fig. 2). Although we were not able to quantitate the amount of such linkage in glutenin polymers, my impression is that this type of connection is likely to be predominant. Thus, these results also support the likelihood that, of the five cysteines in the N-terminal domain of y-type subunits, only one seems prone to form intermolecular disulfide linkages whereas the other four must form two intramolecular disulfide bonds.

The results of Tao and co-workers (6) indicate that the "circularizing" linkage in the Dy10/Dx5 hybrid monomer is apparently representative of the intermolecular disulfide bonds that link N-terminal domains of y-type subunits with C-terminal domains of x-type subunits in normal glutenin polymers. (The end-to-end linkage of the hybrid monomer might be considered a "pseudo intermolecular disulfide bond.") The hybrid subunit was incorporated substantially into both the monomeric form

and high molecular weight polymers in transgenic wheat (5). It is likely that intramolecular disulfide bonds form more rapidly than intermolecular disulfide bonds. The favorable rate at which the hybrid subunit can "circularize" relative to the rate at which it reacts with other subunits to form polymers, however, supports the likelihood that intermolecular disulfide-bond formation between x-type C-terminal domains and y-type N-terminal domains also occurs rapidly when wild-type subunits form glutenin polymers. There is some evidence that intermolecular disulfide-bond formation may slow as polymers form (7,8), perhaps because the rate of diffusion decreases as polymer size increases with consequent decrease in the rate of effective collisions for disulfide-bond formation. The hypothetical time frame for these processes is illustrated in Figure 3. The question of the role of chaperones and disulfide isomerases might be raised at this point, but I know of no clear evidence that they are involved in glutenin polymer formation, and it is at least possible that they do not play a significant role in the process.
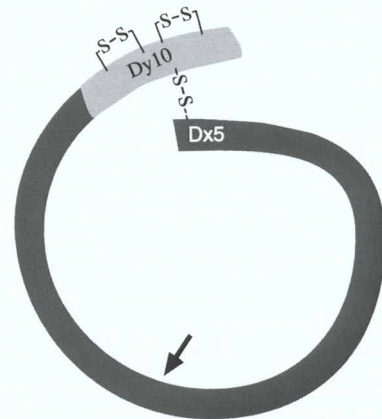
Fig. 1. Circular form of the Dy10/Dx5 hybrid, which has 842 amino acid residues in total. Disulfide bonds are indicated only to give an indication of the number of cysteines in the N-terminal domain (light gray) and in the C-terminal domain (dark gray, but not differentiated from the repeating sequence domain). The arrow indicates the endoproteinase Lys-C site that was cleaved to show the N- to C-terminal connection as described by Shani and co-workers (8).

Disulfide Linkage Between X-Type and Y-Type HMW-GS Is an Important Feature of Glutenin Polymers

Partial reduction studies yield a high level of dimers consisting of x-type to y-type linkages (9,10,11). Although it cannot be ruled out that the x-y pairs produced by partial reduction represent preferential release (8) rather than predominance in glutenin polymers, it remains possible that they are an important feature of glutenin polymers in bread wheats and may indicate that this particular x-to-y linkage occurs rapidly after protein biosynthesis. A rapid formation of x-y linkages may be the basis for the proposal that the HMW-GS form the "backbone" of glutenin polymers consisting of such linkages (9) from which branch chains of low molecular weight glutenin subunits. Although, in the polymerizing system of high and low molecular weight glutenin subunits, it is unlikely that HMW-GS are incorporated solely into a backbone, rapid kinetics for the x-to-y linkage may produce this arrangement de facto. It should be noted, however, that partial reduction studies did not define which domains linked x subunits to y subunits. The findings mentioned above, however, point toward the x-type C-terminal domain being linked to the y-type N-terminal domain. Köhler and co-workers (12) found that y-type subunits can link with other y-type subunits by means of two disulfide bonds in each of the N-terminal domains, which may be in conflict with the above hypothesis. It is necessary, however, in future work to determine the proportions of the two types of linkages in glutenin polymers.

The Dx5-Type Subunits Primarily Responsible for the Stronger Dough Characteristics of Wheats Having the Dx5 + Dy10 Pair

It is generally accepted that a cultivar with the HMW-GS pair Dx5 + Dy10 (5+10 pair) will have stronger mixing characteristics than one having the Dx2 + Dy12 pair (2+12 pair) (2,13). There is little structural difference between the Dy10 and Dy12 subunits (14) and none that appears likely to be responsible for different behavior when the subunits are incorporated into glutenin polymers. Accordingly, it seems likely that the difference in dough properties associated with the 5+10 and 2+12 pairs has its basis entirely in the structural difference between the x-type subunits, specifically a fourth cysteine residue in the Dx5 protein located near the N-terminus at the start of the repeat region (15, 16). Gupta and co-workers (1996) noted that a biotype expressing 5 + 10 accumulates larger polymers earlier than a biotype expressing 2 + 12, which would be in accord with the more rapid formation of such polymers in cultivars expressing 5 + 10 pair.

Dx-5-Type Subunits Cause the Polymer Chain To Branch

Dx-Type Subunits. HMW-GS Dx2 and Dx5 are identical in amino acid sequence through their first 100 residues, with one exception: there is a serine at residue 97 in Dx2 that has mutated to a cysteine residue in Dx5. This fourth cysteine in Dx5 is located at some distance from these first three cysteines. The arrangement of cysteines is illustrated in Figure 4, based on the proposed structure described by Köhler and co-workers (17), which shows the structure of Dx5 in the N-terminal region through 114 residues (both Dx5 and Dx2 have more than 800 amino acid residues in their complete molecules). The structure of Dx2 in this region can be understood from the structure of Dx5. Both Dx5 and Dx2 presumably form one intermolecular disulfide bond by way of their C-terminal cysteines. (There is only one in each subunit.) They are likely, however, to form more than one intermolecular disulfide bond by

way of their N-terminal domains (17,8). Furthermore, the four cysteines near the N-terminus of Dx5 make it more likely that Dx5 will, on the average, form more intermolecular disulfide bonds than Dx2, which has only three cysteines near the N-terminus. Nevertheless, the degree of branching has not yet been established for either subunit.

If we consider only the N-terminal regions, when Dx2 or Dx5 polymerize through disulfide-bond formation, the initial cross-link will probably develop rapidly in either subunit. However, in Dx5, this first cross-link might be formed from any one of the first three cysteines (considering them as a group) or from the fourth cysteine, which is considerably removed from the first three (Fig. 4). Thus, in Dx5, formation of a second disulfide cross-link involving a third approaching protein subunit should occur rapidly as there would be little steric hindrance to a second oxidation that would be most likely
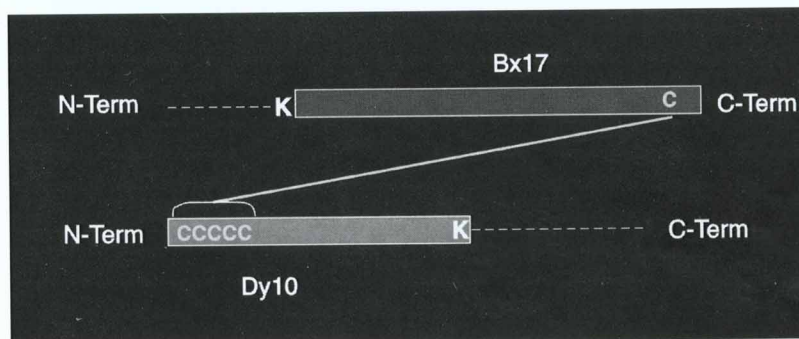

Fig. 2. Schematic of peptide as described by Tao and co-workers (6) illustrating linkage between Dy10 N-terminal and Dx17 C-terminal domains. K (lysine) indicates endoproteinase Lys-C sites that were cleaved to yield the C-terminal half of Bx-type subunits (top bar) and the N-terminal half of subunit Dy10 (lower bar). Dashed lines represent the missing part of each subunit.
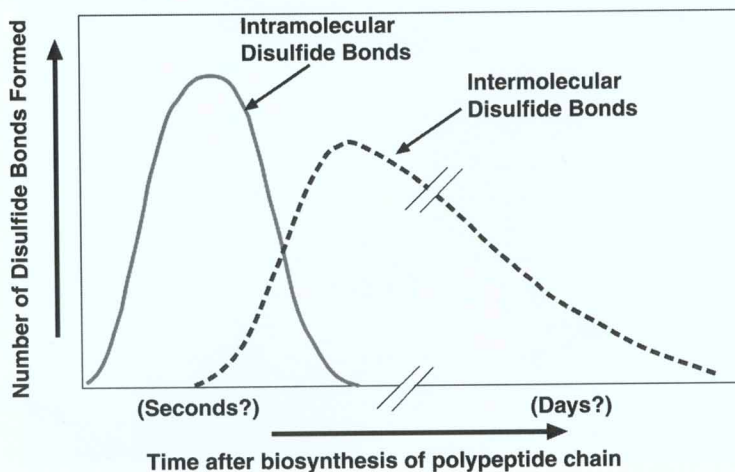

Fig. 3. Hypothetical time frames for intramolecular disulfide-bond formation versus intermolecular disulfide-bond formation. The time frame is discontinuous to indicate that although intramolecular disulfide bonds probably form rapidly within seconds, the formation of intermolecular disulfide bonds may occur both rapidly and more slowly, as polymers grow and the rates of molecular diffusion and rotation decrease.

to occur at the remaining distant site. In Dx2, however, once formation of an intermolecular cross-link has occurred, steric hindrance will occur upon the approach of another glutenin subunit to one of the remaining two cysteines (Fig. 4) in the somewhat tightly clustered group of three (note residues 1–40 in Fig. 4). The spatial arrangements and potential steric hindrance referred to above may be surmised from viewing the relative dimensions of the molecule and distances in the molecular model of the N-terminal domain of Dx5 shown in Figure 4, which is based on the proposed structure as described by Köhler and co-workers (17). The α helical region predicted for residues 5-32 (17) would definitely interfere with

formation of an intramolecular disulfide bond between cysteines 10 and 25 and possibly interfere with formation of an intramolecular bond between cysteine 40 and either one of cysteines 10 and 25, but the situation is less clear in the latter case. Nevertheless, the results of Shani and co-workers (8) suggest that the N-terminal region of subunit 2 can form more than one intermolecular disulfide bond, although it would be desirable to have additional evidence supporting this in the future.

The steric hindrance postulated for the region containing the first three cysteines does not mean that the remaining cysteines of this group will not eventually form disulfide bonds. Further reaction may occur on occasion, perhaps even with low mo-

lecular weight thiol compounds, such as glutathione (18), but reaction of cysteines with those on other large molecules (proteins) is likely to be slowed. If, by way of its N-terminal domain, Dx5 forms two or more intermolecular disulfide bonds quite rapidly because of a much lower degree of steric hindrance, which would give rise to a greater frequency of effective collisions, this would make Dx5 more effective than Dx2 as an agent of polymerization and chain branching during formation of glutenin polymers. This implies that the final thermodynamic states do not differ greatly in free energy, allowing the kinetic factors to predominate. The circumstantial evidence then points toward chain branching as a possible explanation for the tendency of varieties containing Dx5 to have stronger dough mixing characteristics than varieties with Dx2.

Barcelo and co-workers' finding (19) that overexpression of Dx5 in a transgenic wheat line strongly increased dough strength also supports the likelihood that differences between 5 + 10 and 2 + 12 wheat cultivars lie with the x-type subunits. If overexpression of a Dx5 subunit is combined with rapid formation of disulfide bonds, however, the resulting polymers might acquire a rather different "shape" as a consequence of branching combined with an increased number of nucleation sites for polymer growth.

Bx-Type HMW-GS Are Unlikely to Cause Branching. Although Bx7, for example, has the usual three cysteines in the N-terminal domain, the first two (residues 10 and 17) are closer together than those of Dx5 and Dx2 and have been found to form an intramolecular disulfide bond (17). A model of the N-terminal domain of Bx7 is shown in Figure 5. In the model, the disulfide bond between residues 10 and 17 leaves the remaining cysteine at the N-terminus, along with one cysteine at the C-terminus, free for intermolecular disulfide-bond formation. Consequently, the absence of the necessary three or more cysteines available for intermolecular disulfide-bond formation would not allow branches to form from subunit Bx7. This is presumably true for other Bx alleles, such as Bx17, as well. They will extend a chain in a "linear" fashion.

Accordingly, I propose that, in general, linear polymers contribute less to dough strength than do branched polymers. The argument for the relationship of a higher level of branching to increased dough strength stems largely from the circumstantial evidence connecting Dx5 to greater strength. It appears likely that Bx7 and equivalent allelic forms would tend to decrease dough strength more than the Dx types in a bread wheat line or cultivar because, even though Dx2 is likely to branch less than Dx5, branching cannot be ruled out for Dx2. (See discussion in Shani and co-workers [8].) Doughs with higher ratios
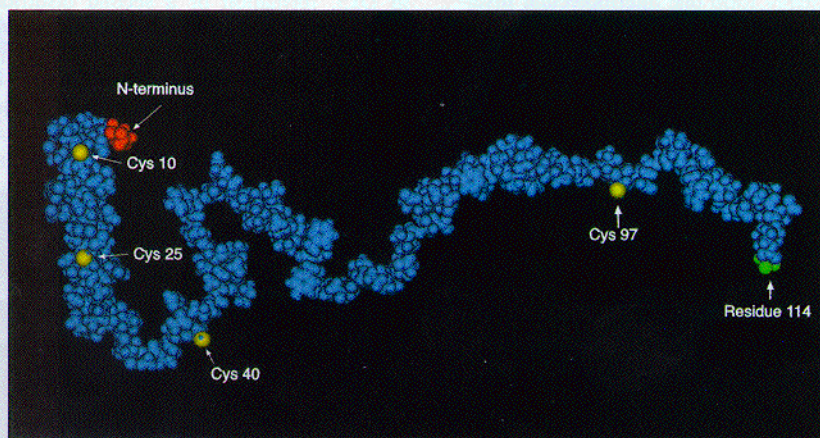
Fig. 4. N-terminal region of Dx5 (residues 1–114) showing cysteine positions. Computer modeling is of molecular structure described by Köhler and co-workers (17). The polypeptide chain of the complete molecule incorporates 827 amino acids. Colors are arbitrarily chosen to illustrate cysteine positions and the N-terminal and C-terminal (of the illustrated 114-residue peptide) amino acids.
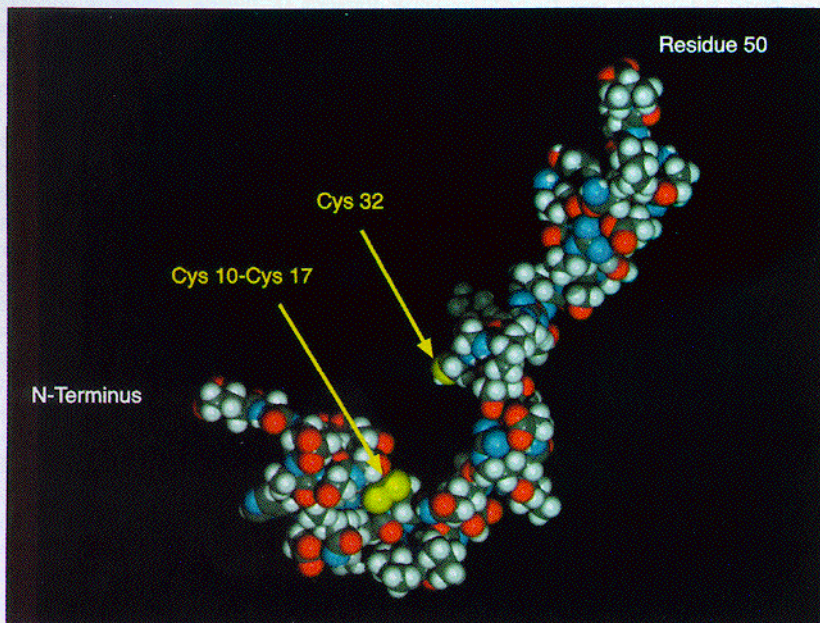
Fig. 5. N-Terminal region of Bx7 (residues 1–50) showing arrangement of cysteine residues and a single intramolecular disulfide bond. Computer modeling is of molecular structure described by Köhler and co-workers (17). The polypeptide chain of the complete molecule incorporates 772 amino acids. This model is colored in traditional CPK atom colors—hydrogen, white; carbon, black; nitrogen, blue; oxygen, red; and sulfur, yellow.

of nonbranching subunits might show increased extensibility with fewer negative effects on properties than, say, would increasing the ratio of gliadin monomers to glutenin polymers (20). As a consequence of its "shape," a linear polymer of a given molecular weight might actually be better able to increase extensibility without sacrificing dough integrity than would a branched polymer of the same molecular weight. On the molecular scale, glutenin polymers are small relative to the starch granules, and overlaps are then crucial in maintaining the stability and elasticity of the protein matrix surrounding the granules (21). The glutenin polymers must physically overlap one another in order to facilitate formation of the hydrogen bonds and other secondary interactions that will stabilize the protein-protein interactions.

Increased Length of the Repeating Sequence Domain in HMW-GS Correlates with Increased Dough Strength

D'Ovidio and co-workers (22) constructed novel HMW-GS genes in which the size of the Dx5 repeat region was varied. Mixing tests in which the proteins (obtained by expression in *E. coli*) were incorporated into doughs indicated that increase in the length of the repeating sequence domain led to stronger dough mixing characteristics (23). Accordingly, although it is not yet possible to quantitate this effect, it appears likely that the longer the repeat domain, the greater the contribution of the subunit to dough strength or elasticity. Although this was demonstrated with the repeating sequence of an x-type HMW-GS, I shall assume that my conclusion also applies to the repeating sequence domains of y-type subunits and of LMW-GS. The LMW-GS have a different type of repeat (24), but I think that the predominance of glutamine (with its strong tendency to participate in hydrogen bonding interactions) in all types of repeats makes it likely that longer repeating sequence domains will be more effective in strengthening a dough than shorter domains, regardless of the repeat type or whether the repeat is part of a HMW-GS or a LMW-GS.

LMW-GS Form Linear Polymers and Contribute to Dough Characteristics in Durum Wheats

Durum wheat cultivars generally have a smaller proportion of HMW-GS relative to LMW-GS than bread wheat cultivars, largely as a consequence of the absence of the D genome, which contributes a major pair of HMW-GS in most bread wheat cultivars. The LMW-GS of durum wheat cultivars have shown stronger correlations with pasta-making quality than the HMW-GS (25), and doughs from durum wheats tend to be less strong in comparison with those of bread wheats. The importance of LMW-GS is likely to result in part from their quantitative predominance. Masci and

co-workers (24) have suggested, on the basis of LMW-GS structure, that most LMW-GS subunits will form linear polymers, having only two cysteine residues capable of forming intermolecular disulfide bonds, one located at the N-terminal end and the other located near the C-terminal end of the protein. The lesser strength of durum wheats, as a consequence of the predominance of LMW-GS forming linear polymers, would be in accord with my suggestion that linear polymers contribute less to dough strength than branched polymers. Of course, the absence of the Dx HMW-GS from tetraploid durum wheat cultivars also tends to decrease dough strength insofar as these Dx subunits seem to be the most important types contributing to branching of glutenin polymers. The Bx-type HMW-GS, which are fairly commonly found in durum wheats, also contribute to formation of linear polymers. Furthermore, the lengths of the repeating sequence domains of LMW-GS, which are much smaller than those of HMW-GS, may also contribute to lesser dough strength in durum wheat.

Although LMW-GS apparently form linear polymers, there is evidence that these polymers are not necessarily smaller in size than polymers that include HMW-GS(26). Gao and Bushuk (26) found that a null line that had no HMW-GS showed very weak resistance to extension. This also supports the possibility that linear polymers contribute less to dough strength than branched polymers and that the effect is not the consequence of degree of polymerization. It remains a difficult experimental task to measure actual molecular weight distributions of glutenin polymers, however, and the molecular basis for the suggested importance of branched polymers over linear polymers in increasing dough strength is not clear. It is at least conceiv-

able that branching may not be directly related to increased dough strength, but rather is artifactually correlated by way of an enhancement of polymerization by Dx5 (and to lesser extent Dx2) that leads to a shift in the molecular weight distribution. Further investigation of this question is definitely needed.

Important Dough Characteristics Relate to the Molecular Weight Distribution of Glutenin Polymers

The molecular weight distribution (Fig. 6) of gluten polymers is fairly certain to be a key factor in the variations of dough strength and elasticity that are commonly observed (20,27). The greater the average length of the glutenin polymers, the more they can overlap, interacting to form a continuous matrix surrounding the large starch granules in a flour-water dough. Correlations between the amount of insoluble (or unextractable) protein in a flour and quality characteristics (28) can also be explained by differences in molecular weight distribution. If glutenin polymers become insoluble when the polymer molecules become larger than some not-yet-defined size, then as the molecular weight distribution shifts to higher range, the quantity of protein above the solubility cutoff increases, as indicated in Figure 6. (However, the very sharp cutoff is meant to illustrate the concept, and the extractability is probably not that well defined in practice). Consequently, as discussed above, the conclusions of Gao and Bushuk (26) that LMW-GS form polymers of equivalent size to those that also include HMW-GS might be only approximately correct. Further experimental work on complete molecular weight distributions (as opposed to measurements in which the distribution is cut off, degraded polymers are measured, or significant amounts of polymers have not been solubilized) is needed.
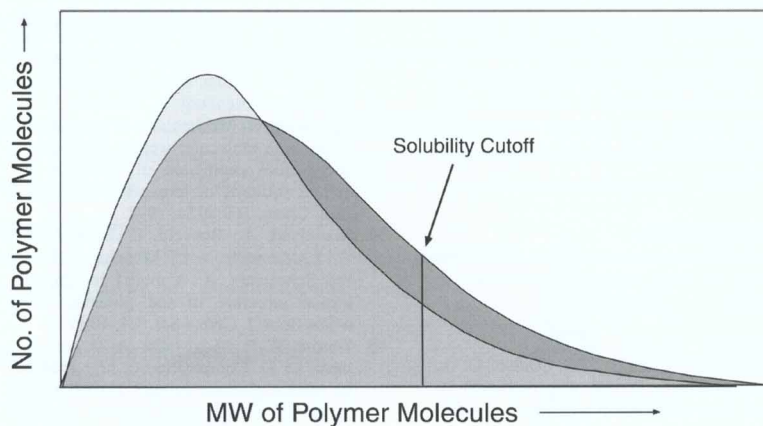

Fig. 6. Two different hypothetical molecular weight (MW) distributions for glutenin polymers (light gray = low MW; dark gray = high MW) with indication of the effect on the amount of insoluble protein resulting from the shift in polymer molecular weight distribution. The molecular weight cutoff for solubility/extraction is also hypothetical and arbitrary, as it has not been determined or calculated as far as I know.

Conclusions

By combining the above observations, I place the glutenin subunits in a hierarchical arrangement that represents their relative potentials for contributing strength to a dough, as measured by its mixing curve (Fig. 7). The basis for the arrangement is mainly a combination of two factors relating to a) the potential of a subunit to form branched polymers, as opposed to linear polymers, and b) the length of the repeating sequence domain. (Branching is better—leading to stronger doughs. Longer repeating sequence domains lead to stronger doughs.) Obviously, quantitative effects will also be superimposed on the intrinsic potentials. For example, a wheat cultivar having "normal" levels of HMW-GS 5 + 10 but high levels of the 42K LMW-GS found in both bread and durum wheat (24) might well be more mellow (or less elastic) in mixing characteristics than a similar cultivar having normal levels of the 42K LMW-GS.

Finally, the role of chain-terminating subunits, such as γ-gliadin-like glutenin subunits that have an extra cysteine in their molecular complement of cysteines—a cysteine that is capable of forming only a single intramolecular disulfide bond, or D-subunits having a single cysteine should not be ignored (29,30). There is not yet evidence that variations in the amounts of such subunits contribute strongly to the variations in quality characteristics observed in different bread wheat cultivars, but such a possibility cannot be ruled out.
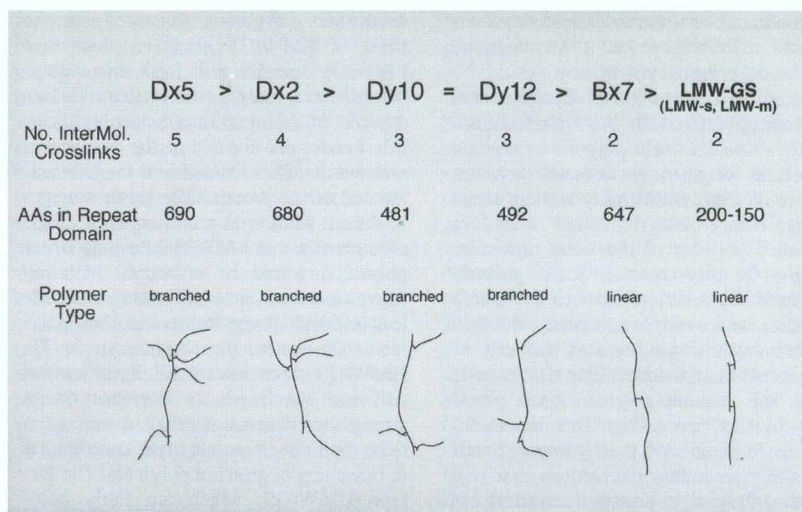
My proposed ranking system might be considered more in the realm of an educated guess than solid theory, but I think the general directions derive from moderately good experimental evidence. The question of the relative importance of repeat domain length versus potential degree of branching reveals one rather weak point in the hypothesis. Does the potential for branching connections at the C-termini of Dy10 and Dy12 subunits take precedence over the greater repeat length of Bx7 in contributing to dough strength? This remains to be seen, and the assignments for these subunits in Figure 7 are rather arbitrary. My intent is to provide a basis for some hypotheses that can be tested in future research.

	Dx5 >	Dx2 >	Dy10 =	Dy12 >	Bx7 >	LMW-GS (LMW-s, LMW-m)
No. InterMol. Crosslinks	5	4	3	3	2	2
AAs in Repeat Domain	690	680	481	492	647	200-150
Polymer Type	branched	branched	branched	branched	linear	linear

Fig. 7. Hierarchical arrangement of high molecular weight- (HMW) and low molecular weight glutenin subunits (LMW-GS) in relation to their proposed intrinsic contributions to dough strength. Indicated are the potential number of intermolecular cross-links per molecule, the number of amino acids (AAs) in the subunit repeating sequence domains, and the type of polymer chain introduced by a subunit (branched versus unbranched). The last characteristic is indicated in a schematic way at the bottom of the figure.

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