Unfolding Gluten: An Overview of Research on Gluten

ω-gliadins
β_{1-4}
γ-gliadins
α_{1-2}
α-helical

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Wheat gluten has remained a subject of investigation since Beccari first reported its discovery in 1728. He described the material he isolated from hand-washed wheat flour dough as a glutinous matter, as opposed to an amylaceous, soluble matter. He identified the isolated glutinous matter as urinaceous in nature based on its decomposition properties. This discovery was significant because up to that point it had been believed that what we now know as protein, referred to by Beccari as "urinaceous spirits," was a substance that was only present in animal products. Beccari also observed that once isolated, the glutinous matter failed to mix further with water and had unique physical properties (2).

The next major breakthrough in wheat gluten research was achieved in 1893 when Osborne and Voorhees developed the now commonly used Osborne fractionation procedure (34). Wheat proteins were separated into four fractions based on differing solubility in the classic four solvent system: water, dilute salt, 70% ethanol, and dilute acid/alkali, which corresponded with albumins, globulins, prolamins, and glutelins, respectively (34). None of the proteins individually could correctly be classified as gluten. However, the combination of prolamin and glutelin (in wheat prolamins and glutelins are called gliadins and glutenins, respectively) yielded the product discovered by Beccari nearly two centuries earlier.

The third major discovery that changed our understanding of gluten was made in 1936 when Balls and Hale (1) published a paper describing the breakdown of gluten upon addition of reducing agents. The authors examined the loss of gluten integrity in the presence of compounds such as cysteine and glutathione. They were unable to definitively identify the phenomenon and thought it might be related to the activation of an enzyme that cleaved disulfide bonds. The inadvertent key finding was the recognition that disulfide bonds are necessary to the structure and functionality of gluten.

Sullivan et al. (45) were less equivocal about the role of disulfide bonds in gluten functionality. This research group came to the conclusion that oxidizing and reducing agents act directly on disulfide bonds among gluten proteins, as opposed to being mediated by a disulfide-cleaving enzyme. Most importantly, they recognized disulfide bonds as being a key factor in the characteristic rheological properties of gluten. This greatly transformed our understanding of gluten and how it behaves in dough systems.

These three key discoveries, occurring in three different centuries, were vital to our initial understanding of wheat gluten. The pace of research in this area greatly accelerated following these breakthroughs and has propelled us into the 21st century and to our current understanding of gluten. Nevertheless, although much scientific research has been conducted on wheat gluten over the past three centuries, we are still far from unfolding these proteins.

Gluten Proteins

Although wheat gluten, which is a water-insoluble storage protein, comprises a smaller part of the endosperm (≈7–20%), it accounts for 85% of the endosperm protein (35). Gluten serves as the primary carbon and nitrogen, and to a lesser extent sulfur, source for the immature plant (37). Glutamine is the most abundant amino acid and together with proline and glycine accounts for >50% of the amino acid residues in gluten (17). This can create limitations in the types of secondary structural conformations that are possible for gluten, especially if the three amino acids tend to be located in conserved positions in repeating motifs. Specifically, type II β-turns ($i \rightarrow i \pm 3$ hydrogen bonding), and to a lesser degree type I β-turns, are likely to be the most favored conformation when an x-proline-glycine-x motif is abundant (16,39,46,47).

Additional distinguishing characteristics of gluten include a relatively large number of hydrophobic amino acid residues (≈35%) and a low charge density due to a relative lack of basic amino acids and the presence of the amide form of acidic amino acids. Methionine, tryptophan, and lysine are the nutritionally limiting amino acids in gluten (10,17,26,37).

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The Osborne fractionation procedure was a step forward in the identification of the two major protein groups comprising wheat gluten. Developments in electrophoresis led to this discovery and further clarified that gliadins and glutenins are two broad groups of proteins, each composed of several individual subunits.

The first well-controlled electrophoretic study to demonstrate that wheat gluten is composed of more than two distinct proteins was performed in 1959 (19). Two fractions were tentatively attributed to glutenin and four to gliadin using moving boundary electrophoresis. In 1961, Woychik et al. (50) were the first to clearly fractionate gliadin into individual subunits. They reported the presence of α-, β-, γ-, and ω-gliadins, with additional subunits in the α- and β-gliadins ($\alpha_{1\text{-}2}$ and $\beta_{1\text{-}4}$, respectively).

Since this work was completed, we now know the four fractions (α, β, γ, and ω) range in molecular mass from ≈15 to 60 kDa and account for ≈50% of gluten proteins (26,30). The α-, β-, and γ-gliadins are referred to as sulfur rich (≈23–35 Cys + Met residues/100,000 g of protein) and generally range from 25 to 40 kDa (10,26). The ω-gliadins are considered to be sulfur poor (≤11 Cys + Met residues/100,000 g of protein) and have molecular masses that are roughly twice those of the other gliadins (10,26). All gliadins are assumed to exist as globular monomers in their native state due to low charge densities and intramolecular disulfide bonds (30). The proline, glycine, and glutamine residues, which comprise a large majority of the amino acids, are distributed in a manner that largely results in an aperiodic structure; however, short runs of an α-helical structure are able to form in areas poor in these three amino acids, with the end result being a structure that can be up to 33% α-helical (26).

Jones et al. (19) first identified two tentative glutenin fractions and four gliadin fractions using moving boundary electrophoresis, whereas Woychik et al. (50) identified only one glutenin band using starch gel electrophoresis. Nielsen et al. (33) examined the heterogeneity of glutenin further by splitting disulfide bonds with performic acid. Sedimentation analysis demonstrated that glutenin is a polydisperse system with average molecular masses ranging from 35 to 150 kDa.

Work since then has shown that the two main glutenin fractions are low molecular weight glutenin subunits (LMW-GS) and high molecular weight glutenin subunits (HMW-GS). Molecular weight estimates for the polymeric glutenin complexes range from 100 to >10 million (49). Estimates place the molecular weight of HMW-GS at ≈70–136 and that of LMW-GS at ≈20–45 after reduction of intermolecular disulfide bonds (8,41), which is in line with the initial findings of Nielsen et al. (33). The sulfur-poor HMW-GS consist of a mix of aperiodic and α-helical structures at both termini, with a β-spiral central repetitive region and two to five free Cys residues (22,31,37,38). The sulfur-rich LMW-GS are not as well characterized as the HMW-GS but are thought to contain an α-helical structure at the C terminal, with irregular β-turns near the N terminal and high flexibility in the central repetitive region; one or two Cys residues are free to participate in intermolecular disulfide bonds (8,20).

Gluten Models

It is the interactions between gliadin and glutenin in the presence of water and energy that result in the formation of what is generally known as gluten. Glutenin and gliadin each contribute different rheological characteristics to dough once a dynamic structure is formed. The gliadin is a filler that contributes to extensibility, while the glutenin network contributes to elasticity. In the context of a dough, especially bread dough, gluten is said to form a continuous network, and it is this network that is responsible for the unique viscoelastic properties of wheat flour dough. Several models have been proposed over the years to describe the basis of the gluten network structure that allows for such unique rheological characteristics.

Ewart (11–13) proposed that the random linking of glutenins by interchain disulfide bonds in a head-to-tail fashion defines the rheology of the gluten network, especially its elasticity. The model, however, does not indicate a specific role for gliadins. Nevertheless, the recognition that polymeric glutenin is the key component responsible for the continuous nature of the network and its elastic properties has furthered our understanding of gluten.

Kasarda et al. (21) alternatively proposed that glutenins form only intrachain disulfide bonds and that noncovalent interactions between molecules result in the creation of a gluten network. These noncovalent interactions are dependent, however, on the formation of specific structural conformations by the glutenins upon intramolecular disulfide bond formation.

Khan and Bushuk (23) proposed a hybrid theory whereby glutenin is capable of forming both inter- and intramolecular disulfide bonds. They identified two glutenin fractions: a lower molecular weight fraction that forms strong noncovalent bonds and a higher molecular weight fraction that forms disulfide bonds. Intermolecular disulfide bonds allow for the development of a continuous polymeric network, while the stability required for noncovalent interactions is provided by intramolecular disulfide-bonded glutenins.

Graveland et al. (14) proposed one of the final models that preceded the currently accepted model. In this model the glutenins form a highly structured branched polymer with specific nonrandom associations. Glutenins were proposed to engage in both inter- and intramolecular disulfide bonds, with a main chain forming the linear head-to-tail disulfide bonds, as proposed by Ewart (11–13). Additional glutenin subunits with intramolecular disulfide bonds were proposed to form branches on the main chain via additional intermolecular disulfide bonds.

All of the preceding models dealt exclusively with the role of glutenin and disulfide cross-links in the gluten network. The current widely accepted model was described by Shewry et al. (40) in 2001. In this model, HMW-GS form the main backbone via head-to-tail disulfide cross-links, with a small degree of lateral disulfide bonding between HMW-GS chains. LMW-GS also participate by serving as chain connectors or terminators depending on the number (odd or even) of free sulfhydryl groups available for disulfide bond formation. Gliadins interact noncovalently with the HMW-GS chains, effectively serving a modulatory role in terms of glutenin elasticity.

Visualizing models of the organization of the gluten network offers much insight into the origins of the unique rheological properties of wheat flour dough. Further theories have been proposed that refine the currently accepted model of the gluten network. They describe the formation of the gluten network and the interactions between gliadins and glutenins that give rise to the rheological behavior of dough.

The implicit assumption of Belton's (3) loop-train theory of wheat flour dough development is that β-sheet secondary structures (trains) are inherently less elastic than β-turn secondary structures (loops) in gluten protein. As a result, the basis for the increased torque and strain

hardening observed during mixing is a transition from predominately β-turn to β-sheet secondary structures in gluten. The progressive stretching and alignment of the cross-linked gluten network during mixing eventually results in a threshold ratio of β-sheet to β-turn structures that is resistant to further deformation. Mixing beyond this point results in the breakdown of the gluten network, and thus, a decrease is seen in mixing torque and strain hardening measurements.

Hamer and Van Vliet (15) described dough development as the hyperaggregation of glutenin polymers. The three stages of aggregation as originally hypothesized by Hamer and van Vliet (15) include 1) the formation of disulfide cross-links between glutenin subunits in the wheat kernel; 2) aggregation of glutenin polymers via entanglement and hydrogen bonding in the wheat kernel and during early mixing; and 3) supra-aggregation of particles by further entanglements resulting from late mixing and during processing. As adapted by Don et al. (6,7), however, the theory relies on the assumption that glutenin exists as a macroparticulate, referred to as glutenin macropolymer (GMP), and that the gluten network in dough is formed by the aggregation of the breakdown products from GMP.

Singh and MacRitchie (42) postulated that dough development is best explained in the context of an entangled polymer system. The theory relies on physical chemistry and polymer theory to describe the various properties of gluten and dough. In dough, gluten behaves as an entangled polymer via disulfide bonds and actual intra- and intermolecular entanglements. Mixing and hydration result in increased segmental motion of gluten, which encourages the buildup of entanglements to a critical threshold size. At this threshold polymer size, further mixing results in the breakdown of the entangled network. It is also the buildup of an entangled network that gives rise to the typical torque curves recorded for wheat flour dough mixing. Singh and MacRitchie (42) described the elastic properties of dough in terms of equilibrium displacement, i.e., applying stretching force to the gluten network results in the displacement and unfolding of gluten proteins from equilibrium, and the removal of force results in the recovery of the equilibrium state insofar as viscous obstruction by gliadin and new entanglements will allow.

Additional phenomena that are well characterized by the entanglement theory include the mixing speed observations by Kilborn and Tipples (24,25) and overmixing, gluten solubility, grain hardness, dough strength and extensibility, and functionality of modified gluten (42).

Re-envisioning Gluten Models

A key observation based on the research to date is that all the preceding work on gluten network formation and function is inherently based on bread as the "ideal" model system, and gluten quality is typically described in terms of its functionality in bread. Within the context of the diversity of wheat-based products, the question arises as to whether these bread-based visualizations of the gluten network apply to all products, and more importantly, how do they advance the science of gluten as it relates to functionality in diverse wheat-based products? There is a substantial gap in our knowledge and understanding of gluten and its function in wheat-based products other than bread that deserves further attention. For example, are gluten and gluten functionality similar in hard and soft wheats? Are the visualizations of the gluten network similar in these wheat types and are the differences a function of the relative amount of gluten proteins contained in them? Research is underway to explore this information gap by investigating the link between gluten structure and functionality in hard and soft wheats.

In recent years, research on soft wheats has primarily focused on puroindolines. Differences in wheat kernel texture, or hardness, can be attributed to mutations in puroindolines (4,32). Soft wheats retain wild-type puroindoline genes that express these proteins in a more functional form in terms of textural modification properties. Hard wheats, on the other hand, possess mutated versions of these genes, and durum wheats lack them entirely. Puroindolines may impact soft wheat gluten functionality through alterations in puroindoline–lipid–starch interactions, as suggested by Dubreil et al. (9).

Attempts to identify soft wheat quality traits have focused primarily on their impact on cookie and cracker baking quality. The conclusions drawn from studies by Souza et al. (43,44) are that genetic traits such as softness equivalent, milling yield, and sucrose solvent retention capacity (SRC) values should be used to direct the selection of new breeding lines. The implication is that gluten functional characteristics may not be as carefully managed in soft wheats as in hard wheats, perhaps due to the model systems being used as end-use quality determinants (e.g., the quality of sugar cookies is overwhelmingly driven by sucrose and pentosans) and a lack of understanding of soft wheat gluten functionality compared to hard wheat.

The basic building blocks of gluten, or the types and amounts of gliadin and glutenin subunits present, are influenced by both genetics and environment. Hard and soft wheats are inherently different on a genetic level with regard to the types of subunits they are predisposed to express. Environmental conditions, including weather, soil conditions, and agronomic management practices, also influence the expression of gliadin and glutenin subunits, as well as the overall protein content. Together both these factors dictate overall gluten quantity and quality. Once a given wheat crop reaches the miller, however, its quality parameters are blended out to an extent with other wheats, and even wheat types, to achieve a set of target specifications (typically protein and ash contents). Unfortunately, these specifications are poor indicators of flour performance. Protein content serves as a good indicator of gluten quantity, but it indicates nothing regarding gluten quality.

Recent results obtained using a high-shear technique (gluten peak tester [GPT]) to measure gluten aggregation kinetics demonstrate the lack of information provided by protein content specifications. The GPT, utilizing high shear (>71,900 rpm), has been used to differentiate the aggregation properties (torque and time) of gluten in a batter system (8.5 g of flour:9.5 g of 0.5*M* $CaCl_2$). Different Ontario soft red winter wheats with similar protein contents demonstrated significantly different GPT peak torque and time values (Fig. 1). In fact, the GPT peak torque for 69 Ontario soft wheat varieties tested ranged from 17 to 39 BU. Additional differentiation between wheat varieties was observed based on growing location (Fig. 2). Interestingly, a study investigating the source of gliadins and glutenins (in hard versus soft wheats) and the resulting behavior of the gluten when the sources and ratios of gliadins to glutenins were changed showed that the ratio per se did not result in similar aggregation kinetics (29). The results suggest that there are inherent differences within the gliadins and glutenins of hard and soft wheats. Further research is required, and is underway, to determine whether the specific fractions and subunits of these proteins influence functionality and the mechanisms by which these interactions influence the gluten network.

Fourier transform infrared (FTIR) spectroscopy has been used in recent research as a tool to study the secondary structures of proteins in flour and during dough development. Li et al. (27) reported an increase in flour and gluten β-sheet structure upon nonmechanical hydration, while others have described increases in β-sheet structure at the expense of β-turn structure during deformation and mixing of flour or gluten (28,36,48).

In the context of dough mixing, results indicate that hard and soft wheat flours do not share a similar gluten structural evolution over the course of mixing. Hard wheat flour doughs demonstrate structural evolution in line with that proposed by Belton (3) and other researchers, while soft wheat flours do not exhibit a significant change in gluten secondary structure with mixing. These results suggest that current models fail to adequately explain the gluten network in these systems and products.

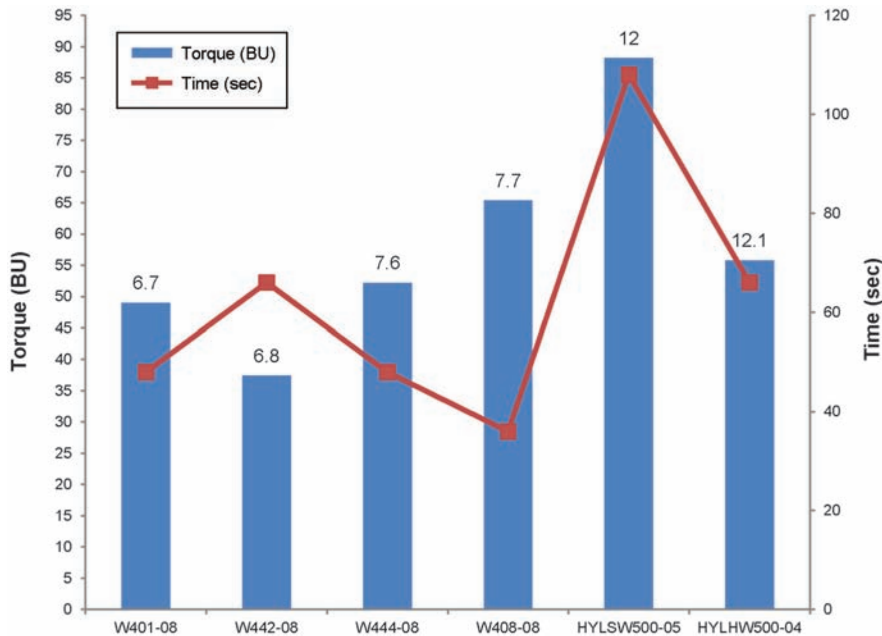

Fig. 1. Gluten peak tester time and torque values for Ontario soft red winter wheat flours with similar protein contents. Values above the bars indicate protein content (14% mb).

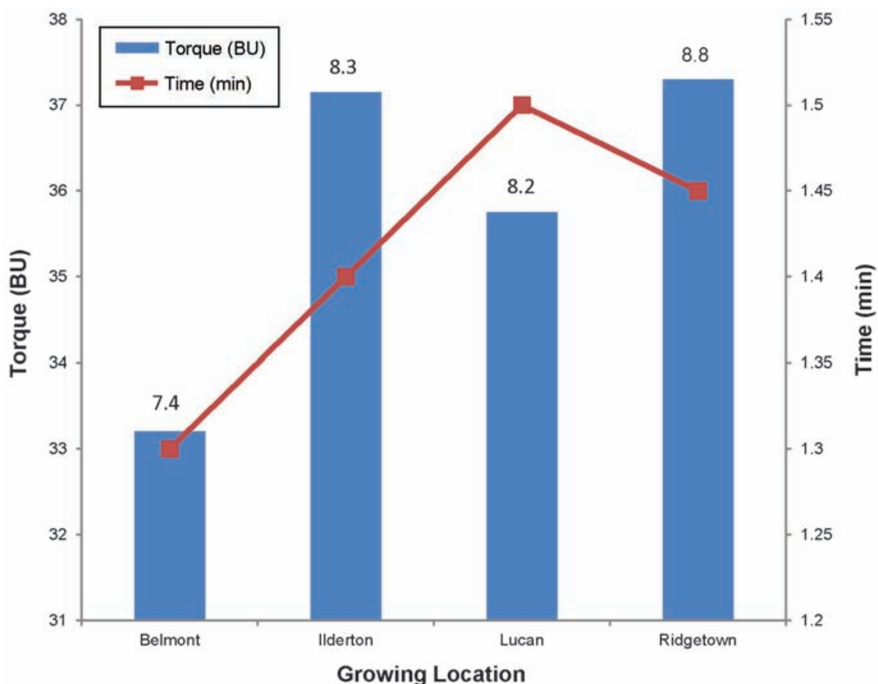

Fig. 2. Gluten peak tester time and torque values for Ontario soft red winter wheat grown in different locations. Values above the bars indicate protein content (14% mb).

Recent research has also focused on the impact of the interactions of other ingredients within a product formulation on gluten secondary structure. The results of a study on the impact of bran on gluten structural changes show that the addition of bran results in gluten secondary structural changes that mimic those of gluten at lower moisture contents due to competition for available water between the gluten and bran. The bran competes more effectively for available water to the detriment of gluten hydration. This has implications for the behavior of gluten in the presence of other ingredients that compete for or restructure water in formulations (5). Information on protein surface hydrophobicity could also provide information on structure that could help predict flour performance.

Research has shown that surface hydrophobicity differs substantially between hard and soft wheat flours, potentially affecting their ability to interact with lipids and other hydrophobic ingredients in formulations (18). The number of hydrophobic sites can vary, as well as the affinity with which the site is capable of binding hydrophobic materials. In the case of hard wheat flour there appears to be a small number of high-affinity binding sites, while soft wheat flour has a large number of low-affinity binding sites (18). The surface hydrophobicity of hard and soft wheat flours also evolves over the course of mixing. Observations using fluorescence spectroscopy suggest that hard wheat flours increase in hydrophobicity, increasing up to peak mixing torque before decreasing to original levels. This parallels the increase in sheet structures and suggests that mixing may serve to unfold and extend hard wheat flour gluten. Soft wheat flour is quite different in that hydrophobicity decreases throughout most of the mixing process, suggesting perhaps that the gluten network in soft wheat is more of an aggregative phenomenon when coupled with the lack of structural changes during mixing.

Conclusions

These observations imply that more information is needed regarding the parameters of gluten quality before a flour can be determined to be of high quality. High-quality flour refers to the ability of gluten to form efficiently and contribute characteristics essential for the target product within the context of its process-

ing environment. Gluten quality is a difficult parameter to evaluate. However, as quality and quantity interact to produce the functionality of the gluten and overall flour, a closer look at structure may provide new insight into and understanding of gluten behavior beyond bread products.

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