

Chapter 1

Gluten: A Balance of Gliadin and Glutenin

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INTRODUCTION

The simplicity with which gluten can be purified from flour by water-washing made it one of the first proteins to be isolated in reasonably pure form. This achievement was first reported by Beccari of Bologna, in 1728 (see Bailey 1941), but it was still many years before the chemical constitution of proteins was elucidated, and before the term “protein” was coined. This occurred in 1838 when Berzelius wrote to Mulder; see Hartley (1951). The term “gliadine” predated even this, being suggested by G. Taddei in 1819, based on the demonstration by Einhof, in 1805, that gluten could be separated into two fractions, based on the extractability of gliadin in aqueous ethanol. The insoluble residue was named “zymom” by Taddei, “plant albumin” by Berzelius in 1826; also “glutin” by de Saussure in 1833 and by Dumas and Cahours in 1843 (Osborne and Vorhees 1893; Wrigley 1993). By the end of the nineteenth century, the terms “gliadin” and “glutenin” were established to describe the two halves of gluten that were extractable and residual, respectively, using 70% aqueous ethanol (Osborne and Vorhees 1893). Significantly, the chemical distinction between these two fractions was demonstrated on the basis of their respective contents of proline and glutamic acid, and the degree of amidation (Osborne and Clapp 1906). Nevertheless, there was the erroneous assumption that each of these components was a pure homogeneous protein.

Researchers in America, Australia, England and France pursued the concept that variations in gluten quality (and thus in dough properties) could be explained by varying the balance between these two major components of gluten. Pursuit of this hypothesis led to the appearance of several conflicting reports of the ratio between gliadin and glutenin in the literature of the late nineteenth century, with values for this ratio ranging from 0.59 to 4.0 (see Tracey 1967). Reasons for these wild variations appear to relate mainly to differences in extraction procedures, but also to difficulties in obtaining reproducible results with extraction as the method of fractionation.

Nevertheless, the results appear to have shown initial promise, with a higher proportion of glutenin relating to dough strength in the words of Guthrie (1896): ‘Flours in which glutenin predominates yield strong, tough, elastic, non-adhesive glutes. Increased gliadin content produces a weak, sticky, and inelastic gluten.’ Today, we would agree with these conclusions, but further research led Guthrie (1912) to abandon this approach: “Further work on this subject has convinced me that the relationship is not as simple as I at first thought; nor is the separation and accurate determination of the two proteins quite satisfactory. This method has, therefore, been abandoned in this laboratory, and is not, I believe, any longer recognised. The question – what is the cause of [dough] strength – still remains to be solved.”

The subsequent century of research in cereal chemistry has revealed the great complexity of the gluten complex, the genetic control of the many component polypeptides, and the importance of the various bonds between the protein chains (see Chapters 2-7). Nevertheless, the concept of a critical balance between the complementary roles of the gliadin and glutenin components is still central to our understanding of gluten function. However, this balance is now more likely to be seen as being due to their distinct contributions to molecular-size distribution, which in turn explains the original distinction of their extractabilities into solution.

GLUTEN: STORAGE PROTEIN AND DOUGH MATRIX

For the wheat plant, the gluten-forming proteins of the grain appear to serve no other role than to provide a reserve of amino acids for the developing seedling when germination occurs. The water-insolubility of these reserve proteins offers the advantage for the plant that the moisture that triggers the germination process will not also cause this storage reserve to leach away. Similar insolubility properties are shared with the storage proteins of other cereal grains (e.g., barley, maize), but they do not provide mankind with wheat gluten’s property of supporting the bubble-forming structure of leavened bread. Thus water-solubility alone does not explain gluten’s unique rheological ability to form the matrix of dough.

Grain ultrastructure and storage-protein biosynthesis

The proteins that will become gluten after milling and dough formation are deposited in the endosperm during grain filling. Their presumed function in the grain is as storage proteins, providing a source of amino acids for the germinating grain. Protein synthesis occurs on the ribosomes (attached to the endoplasmic reticulum) by the translation of the RNA nucleotide sequence, which in turn has been derived (transcribed) from the DNA sequence of the relevant genes on the chromosomes.

Peptide bonds are formed between appropriate amino acids, in the required sequence, by the interaction of messenger RNA and transfer RNAs, each of the latter carrying the appropriate amino acid. Wheat storage proteins do not undergo glycosylation to a significant extent, but a signal

sequence may be removed from the N-terminal of the newly-formed polypeptide by proteolysis, before it is released into the lumen of the endoplasmic reticulum. The important steps of polypeptide folding and disulfide-bond formation probably begin to occur immediately after the formation of the polypeptide chains, within the lumen of the endoplasmic reticulum. Appreciable polymerization continues in the latter weeks of the grain filling process, especially during desiccation (Carceller and Aussenac 1999; Naeem and MacRitchie 2005). The initial polymerization processes (see Chapter 7) appear to be under the control of molecular chaperones,

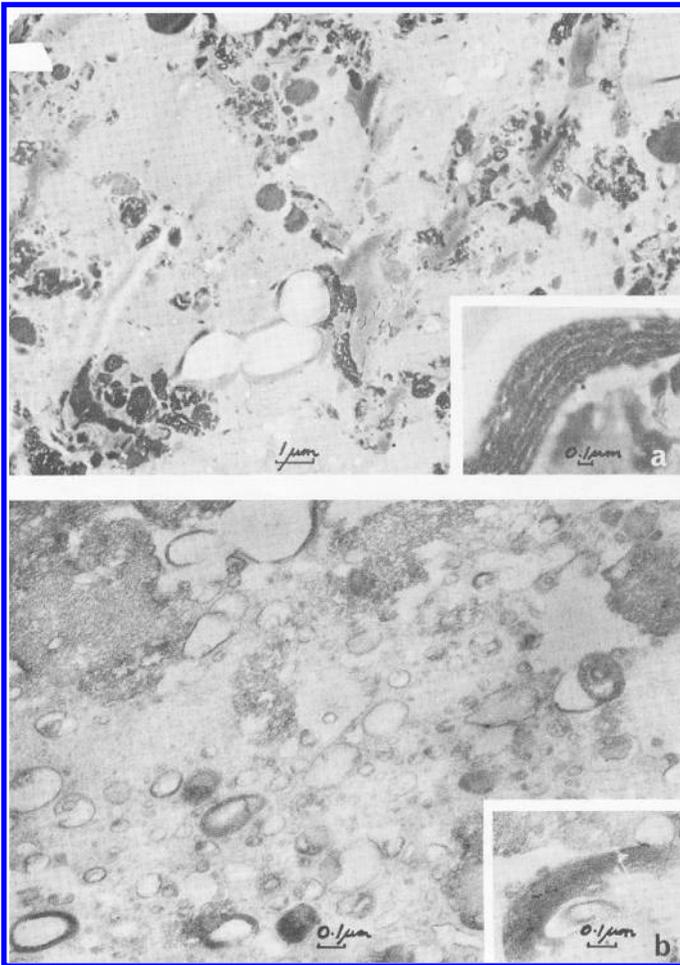


Figure 1. Transmission electron micrographs of cellular organelles in material remaining after extraction with 4M urea of (a) flour particles and (b) gluten. Reproduced, with permission, from Simmonds (1972).

such as protein disulfide isomerase and peptidyl-prolyl *cis-trans* isomerase (Shewry 1999).

The completed proteins are deposited in discrete protein bodies. A group of storage proteins (mainly gliadins) appear to follow a conventional route via the Golgi apparatus to accumulate in Golgi body vacuoles. Others (mainly glutenin polymers) accumulate in the lumen of the endoplasmic reticulum. However, these distinctions are lost during the ripening process as grain moisture decreases and the grain hardens, so that distinct protein bodies are not discernable in the ultrastructure of the mature endosperm.

Nevertheless, some of the ultrastructure of the developing endosperm is visible in flour particles. Figure 1 illustrates membranous structures, detected by transmission electron microscopy, in material from flour and gluten that was insoluble in 4M urea. On the basis of extractability, this material is equivalent to the glutenin fraction. Some of these organelle remnants were identified as endoplasmic reticulum (Simmonds 1972). Some of the protein and lipid of glutenin thus appears to be derived from membrane material involved in the cellular functions of the developing grain. Also detected in flour particles and dough were structures identified as spherosomes, ribosomes and aleurone bodies (Simmonds 1972), providing evidence of the origins of more of the protein and lipid of flour.

Environmental modification of gluten composition

The process of disulfide-bond formation continues in the storage proteins during the ripening (desiccation) of the grain, and it even continues into storage, but at a much slower rate than during grain filling (Wrigley and Békés 1999). This ongoing process is illustrated diagrammatically in Figure 2. Disulfide-bond formation again accelerates during the heat treatment of processing e.g., baking or extrusion. These processes make significant contributions to the degree of polymerization of the glutenin proteins and thus to the molecular-weight distribution of the overall gluten-protein complex (see Chapter 7).

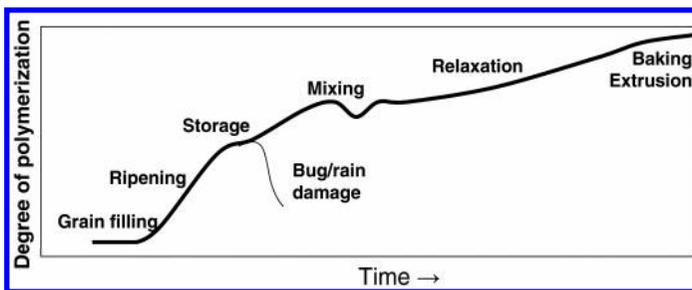


Figure 2. Degree of polymerization of glutenin protein (vertical axis), due to disulfide-bond formation, a process that continues throughout grain filling, due to storage and processing. Adapted from Wrigley and Békés (1999).

The rate of change of molecular-weight distribution depends obviously on the conditions of grain filling and of storage. Abiotic factors, such as hot conditions during grain filling, may alter gluten composition. For example, few days with maxima over 35°C have been reported to reduce the molecular-weight distribution by interfering with the normal processes of disulfide-bond formation, producing weaker-than-expected dough properties (Lafiandra et al 1999). In contrast, hot storage conditions (e.g., some months at over 35°C) may lead to a continuation of disulfide-bond formation, so that the dough formed from this grain may be stronger than it was before storage (Wrigley and Békés 1999). On the other hand, biotic factors may also alter the degree of polymerization, such as the action of a protease (see Chapter 14) left by insects that attack the immature grain in the field (Sivri et al 1999).

Plant nutrition also has the potential to modify gluten function. Most obviously, an abundance of nitrogen fertilizer is likely to increase grain-protein content. However, this phenomenon may be accompanied by a scarcity of sulfur, depending on the type of fertilizer used, leading to a depletion of the sulfur-rich proteins and a higher proportion of low-sulfur proteins, particularly the omega-gliadins, which are virtually devoid of sulfur (Randall and Wrigley 1986). In addition, the proportion of HMW subunits of glutenin is likely to increase at the expense of sulfur-rich proteins (MacRitchie and Gupta 1993). Sulfur deficiency is therefore likely to cause significant changes in dough quality by upsetting the normal balance of gliadin and glutenin proteins, and the balances within each class, i.e., omega-gliadins *versus* other gliadins, and HMW *versus* LMW subunits of glutenin (Zhao et al 1999a; Wieser et al 2004). Quality defects due to sulfur deficiency may increase as wheat growing becomes more intensive, and as N fertilizer application increases (especially for low-S sources of N, such as urea or anhydrous ammonia) without the complementary application of sulphur (Zhao et al 1999b).

DOUGH FORMATION AND DEVELOPMENT

There are three fundamental components of a bread dough, namely, flour, water, and mixing. The effects of other ingredients, yeast, salt, sugar, fat, etc., whose importance is mostly quantitative not qualitative, shall not be discussed. For comprehensive reviews of the subject, see Hosenev and Rogers (1990) and Bushuk (1998a, 1998b).

The wetting of flour

Flour is basically wheat endosperm separated from other wheat-grain components (particularly germ and bran) and ground to a particle size suited to meet the standard of the type of flour, usually fine enough to pass through a 149-micron flour sieve. Examination of flour under a microscope reveals that it varies widely in particle shape and size. Proximate analysis shows that particles also vary widely in composition from 0 to 100% starch,

and 100 to 0% protein (Jones et al 1959). The natural granular condition of the starch can be partly “damaged” during the milling process. The degree of damage plays an important role in dough formation. The main constituents of flour are starch, protein and water (moisture content). In addition, wheat flour contains two minor constituents, non-starch carbohydrates and lipids, both of which contribute to dough formation. Due to variations in physicochemical structure, flour constituents differ in their water-uptake capacity, from 0.3 g/g for granular starch to 10 g/g for the non-starch carbohydrates (Bushuk 1966).

Dough formation begins when water comes into contact with flour. Flour particles that contain protein exude proteinaceous fibrils, which interact (stick together) to form a cohesive dough (Amend and Belitz 1989). The changes that follow during mixing are collectively called “dough development”. It is not fully understood what happens at the molecular level but a likely sequence of events is as follows. First, dough mixing blends the ingredients into a homogeneous mass (at the super-molecular level of structure). At this point, flour particles absorb water at a rate and amount depending on their water-binding capacity and the amount of water added (water absorption). Mixing aids hydration by exposing new dry surfaces on flour particles for interaction with water. Subsequently, further changes occur at the molecular level including interaction of gliadin and glutenin and re-orientation of glutenin via S-S interchange (Tanaka and Bushuk 1973; Graveland et al 1993).

Bonds involved in the gliadin-glutenin balance

It is the balance of gliadin and glutenin that is a fundamental requirement; without both, no dough is formed. During the early stage of mixing, depending on intrinsic dough strength, a dough is not formed even though all the water may have been absorbed. As mixing continues, glutenin interacts with gliadin to form gluten, the viscoelastic matrix of the dough. Gliadin and glutenin molecules of diverse flours differ in the “number” of interacting hydrogen bonds. The rate of interaction depends on the specific surface area of the glutenin (Sapirstein and Fu 2000). The larger the glutenin molecule, the smaller the specific surface area, and the longer the mixing required to achieve full development. The development process can be visualized by recording dough consistency with a Farinograph or a Mixograph. Optimum development of the dough can be achieved only if the mixer can generate a torque above a critical value (Kilborn and Tipples 1972). Best baking results are obtained with doughs that have been mixed just past the maximum in the consistency curve.

Several chemical bonds are involved in the development of the optimum dough structure. (See Bushuk (1998a) for a review.) The most important are the hydrogen and hydrophobic interactions, disulfide bonds and (possibly) crosslinks involving dityrosine (Tilley et al 2001). Hydrogen bonds are much weaker than covalent bonds but, because of the large

numbers that act cooperatively, they contribute significantly to the structure of the dough. Another unique feature of hydrogen bonds is their ability to interchange under stress and thereby facilitate re-orientation of gluten proteins. The evidence for the importance of hydrogen bonds in dough includes the high proportion (about 35%) of glutamine in the gluten proteins, the drastic effect on dough rheology of adding hydrogen-bond-breaking agents (such as urea), and the strengthening effect of heavy water (D_2O) compared to ordinary water (Tkachuk and Hlynka 1968).

Hydrophobic bonds result from the interactions of non-polar groups in the presence of water. Experimental evidence for the presence of these bonds in dough includes the rheological effects of adding organic solvents and soap solutions, and their detection by NMR spectroscopy. Their functionality is similar to that of hydrogen bonds but the overall effect is much smaller. Hydrophobic interactions differ from other chemical bonds because their energy increases with increasing temperature; this could result in increased stability during baking.

Disulfide bonds play a key role in the formation and development of dough. They form strong cross-links within and between polypeptide chains, thereby stabilizing hydrogen bonds and hydrophobic interactions. During dough formation and development, disulfide bonds can be mobilized through disulfide-interchange reactions (Goldstein 1957). The interchange reaction requires a "mobile" (soluble or low-molecular-weight) sulfhydryl-containing substance to initiate the series of disulfide interchanges (Bloksma and Bushuk 1988). The total number of S-S bonds does not change; only their location in the glutenin molecule is altered. The possible contribution of dityrosine bonds to dough structure has been proposed (Tilley et al 2001). Further research is required to confirm this proposal.

Dough formation and development requires a critical balance of constituents, ingredients, and energy input by mixing. The interaction among these three factors is complex. However, the key constituent of the gluten matrix is hydrated protein. As a result of this knowledge, the single figure of protein content is a critical factor in determining the value of wheat in trade. The second factor is protein quality, i.e., how effective is the protein with respect to its role in processing? This question is not so easy to determine as is the estimation of protein content. Obviously, the ultimate test of protein quality is its performance in commercial processing. The prediction of protein quality has long been the topic of intense research. Analysis of gluten composition has been a major avenue of this research.

GLUTEN COMPOSITION

Extraction problems with gluten

Conventional methods of analysing proteins require that they should be dissolved in aqueous solvents. The extraction of gluten proteins is greatly

facilitated by the reduction of all disulfide bonds, such as is done in single-dimension SDS-PAGE or in proteome analysis. However, SS-bond rupture is certain to destroy some of the most important information about gluten composition. Therefore, a search for a universal solvent for wheat endosperm proteins has continued for a long time. The amount of extractable protein is increased by using alcohols (e.g., ethanol and propanol), acetic acid, and urea - used alone or in combination with acetic acid and cetyltrimethylammonium bromide (AUC), SDS and soaps. Nevertheless, with all solvents, a significant amount of protein remains in the insoluble residue (for review see Mifflin et al 1983). This residue has been suspended in dilute SDS solution by introducing energy in the form of ultrasonics, and this is the basis of an assay system that determines the percentage of “unextractable polymeric protein” (%UPP) (Gupta et al 1993a; Batey et al 1991) (Figure 3). This enigmatic name attempts to indicate the part of the glutenin polymer is that is most difficult to suspend. It is thus a measure of the largest glutenin polymers, and these are likely to shift the balance of molecular-weight distribution towards stronger dough properties (Southan and MacRitchie 1999).

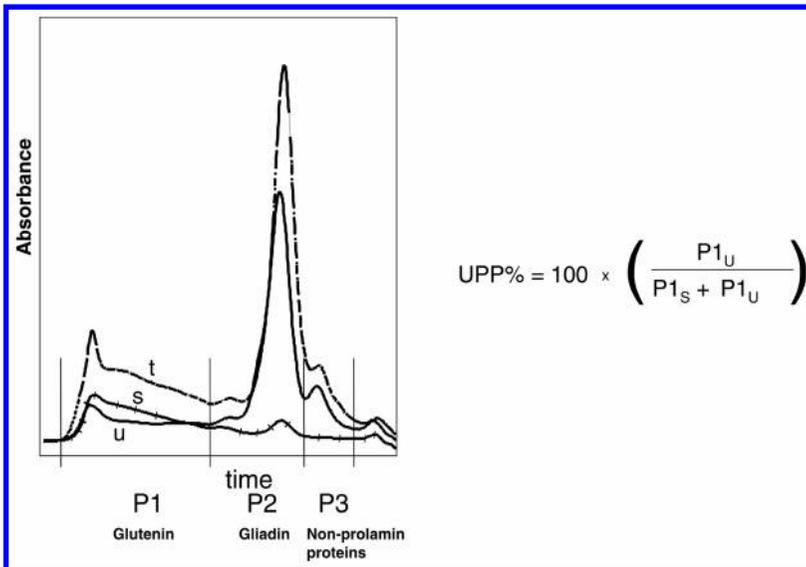


Figure 3. Size-exclusion HPLC of flour proteins. The largest proteins (Peak 1, glutenin) are eluted first. Elution profile “t” is total protein extracted with sonication to help in extraction. Analysis of the percentage of unextractable polymeric protein (“%UPP”) involves SE-HPLC of successive extracts, shown as profile “u” (the first involving no sonication) and a second profile “s” after the use of sonication. These analyses result in the respective peak areas for Peak 1, used in the equation shown.

The use of size-exclusion high-performance liquid chromatography (SE-HPLC) has permitted a re-defining of the traditional Osborne fractions (Figure 3). Osborne's solubility-based fractionation provided poor resolution between fractions. Better resolution is provided by SE-HPLC for an extract that contains virtually all the protein of flour, extracted, without significant rupture of disulfide bonds, into neutral phosphate buffer containing SDS, using sonication to "shake" the largest gluten aggregates free. Thus SE-HPLC has permitted accurate analysis of protein composition in terms of glutenin, gliadin and water-soluble proteins. The determination of %UPP provides the added detail of distinction between glutenin aggregates of modest and of large size range.

The difficulty of having to have the gluten proteins in aqueous solution for conventional fractionation might be overcome by using methods applicable to dough in its water-scarce state. The use of such methods might avoid the introduction of artefactual manipulations involved in protein dissolution, with the consequent risk of information loss. We are still in need of such methods, but directly applicable techniques, such as NMR, NIR and ultrasonics, may help to achieve these objectives.

Traditional methods of fractionation

Wheat endosperm (flour) contains 10-13% protein. The protein is highly heterogeneous in composition and in molecular weight. Research on structure and functionality of wheat proteins begins by extraction with an appropriate solvent and separation into fractions comprising proteins of similar properties. The first comprehensive fractionation of wheat-flour proteins was carried out by Osborne (1924) using sequential extraction by water, salt solution, and 70% ethanol solution. This fractionation formed the basis of the nomenclature of cereal proteins, albumins, globulins, gliadins and glutenins. Attempts to relate these fractions to bread-making quality, which followed, were unsuccessful. The classical Osborne fractionation was subsequently modified to produce five fractions. In the modified procedure, glutenin was separated into two fractions, subsequently called "soluble" and "insoluble" glutenin, by extraction with 0.05M acetic acid solution (Chen and Bushuk 1970).

A significant problem with Osborne fractionation, discovered later (Dupuis et al 1996), is that considerable gliadin remains in the residue after extraction with 70% ethanol solution. This problem was resolved by the use of 50% propan-1-ol to extract the gliadins (Byers et al 1983; Marchylo et al 1989; Fu and Sapirstein 1996; Sapirstein and Fu 1998). This fractionation gives two glutenin fractions, namely, "soluble" and "insoluble". The resulting "soluble" glutenin fraction contains very little gliadin as a contaminant. The "insoluble" glutenin can be extracted after reduction with dithiothreitol. These two glutenin fractions can be analyzed qualitatively and quantitatively for subunit composition by reversed-phase high-performance liquid chromatography (RP-HPLC) (Fu and Sapirstein 1996). For wheat varieties of diverse baking quality, the amount of insoluble glutenin is directly related to loaf-volume potential (Sapirstein and Fu 1998).

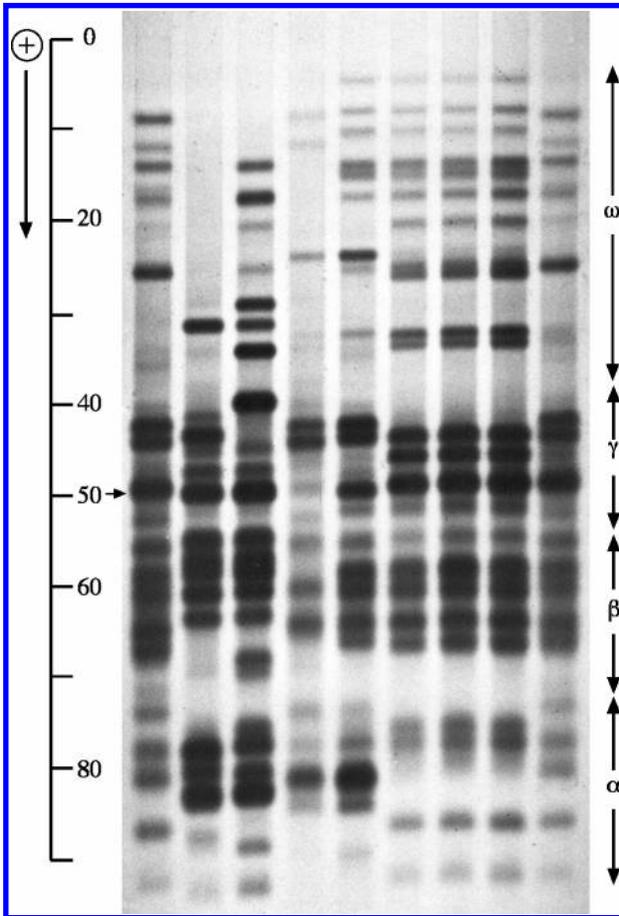


Figure 4. Acidic polyacrylamide gel electrophoresis (A-PAGE) patterns by the method of Bushuk and Zillman (1978) of eight Canadian wheat cultivars showing zones designated by Greek letters according to Bushuk and Sapirstein (1991), and relative mobility based on gamma-gliadin 50 as the reference band. Identity of cultivars (from left) is Marquis (standard), Neepawa, Manitou, Thatcher, Glenlea, Fredrick, Wascana, Wakooma and Marquis. Adapted from Bushuk (1993).

Several methods are available for the analysis of wheat-protein fractions obtained by sequential extraction or by extraction with a single solvent. Monomeric proteins (e.g., gliadins) can be separated and quantified by acidic (pH 3.1) polyacrylamide gel electrophoresis (A-PAGE) (Bushuk and Zillman 1978) (Figure 4). PAGE in the presence of sodium dodecyl sulfate (SDS-PAGE) has been widely used for analyzing the glutenin sub-

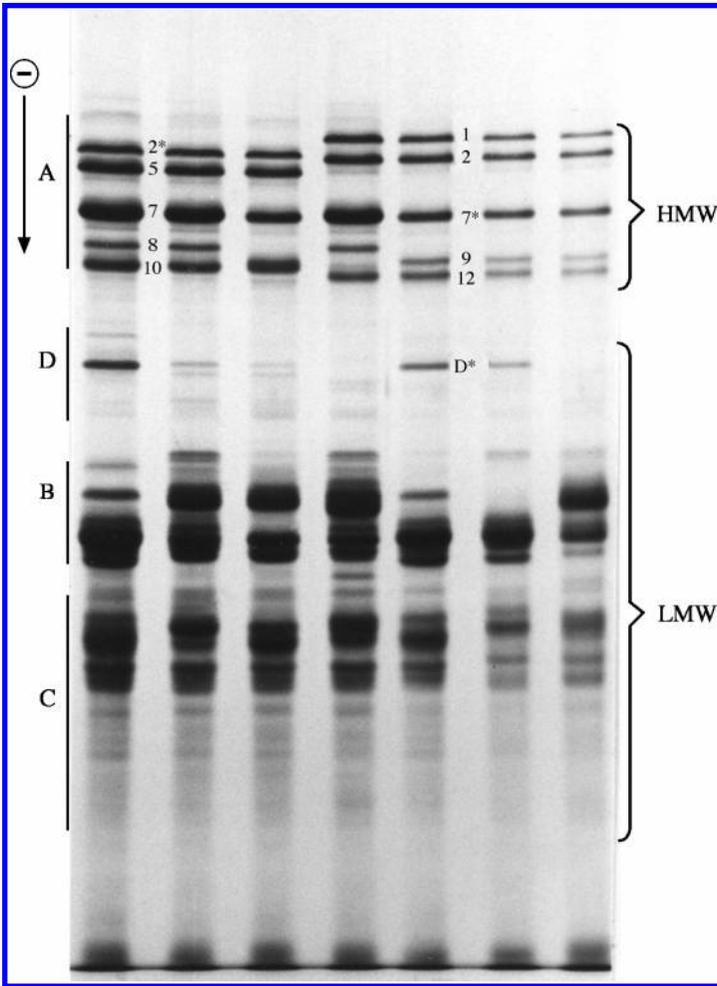


Figure 5. SDS polyacrylamide gel electrophoresis (SDS-PAGE) patterns of reduced 50% 1-propanol-insoluble glutenin of seven Canadian wheat cultivars showing high (HMW) and low (LMW) molecular weight subunits, and mobility zones A, D, B and C. Identity of cultivars (from left) is Harus, SWS-52, AC Karma, Biggar, Katepwa, Roblin and Glenlea. The HMW subunits of AC Karma and Glenlea are numbered according to Payne and Lawrence (1983) and Marchylo et al (1992). The D-zone band for AC Karma and Glenlea is an omega-gliadin. Adapted from Fu (1996).

units obtained by reduction of glutenin fractions (Khan and Bushuk 1977). Figure 5 provides some SDS-PAGE patterns of glutenin subunits. Gel filtration chromatography and RP-HPLC can be used for analysis of unreduced and reduced extracts.

Recent methods of fractionation

Several new methods for separation and analysis of wheat flour proteins have been introduced in the past decade. Flow field-flow (FFF) fractionation can detect the presence in native polymeric glutenin (dissolved in 0.05M acetic acid) of components with molecular masses as large as millions of Daltons (Stevenson et al 2003). This technique should be helpful in confirming the hypothesis that breadmaking quality is directly related to the average molecular mass of glutenin. The second modern technique introduced recently is the matrix-assisted desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Dworschak et al 1998). This technique can resolve proteins with molecular masses of hundreds of kiloDaltons. Accordingly it should be useful for analyzing both gliadins and glutenin subunits.

Proteome analysis, another recent approach to defining protein composition, is an attempt to fractionate and characterize all the polypeptides of a specific tissue. The method usually involves high-resolution two-dimensional electrophoresis of a fully reduced extract of the polypeptides of the tissue. This recent method is based on combined isoelectric focusing and gel electrophoresis. In its first use, two-dimensional gel electrophoresis was applied to gliadin proteins, thereby demonstrating their great heterogeneity (Wrigley 1970). In its use for proteome analysis, the method now goes much further in its

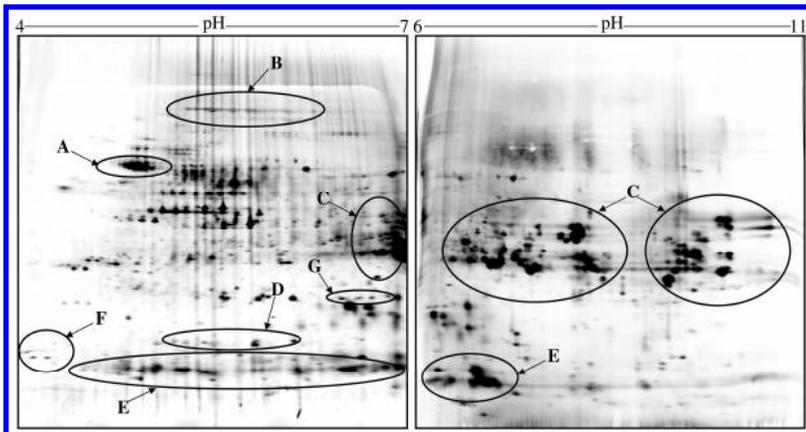


Figure 6. Proteome analysis of the major classes of polypeptides in immature wheat endosperm of the variety Wyuna, fractionated by two-dimensional gel electrophoresis, using pH gradients in the first dimensions of 4-7 (left) and 6-11 (right). Protein-family regions correspond to (A) protein disulfide isomerase, (B) high-molecular-weight glutenin subunits, (C) gliadins, (D) small heat-shock proteins, (E) alpha-amylase/trypsin inhibitors, (F) acidic ribosomal proteins and (G) superoxide dismutase isoforms. Reproduced from Skylas and Wrigley (2004), with permission from Elsevier.

resolution ability, most recently demonstrating that some 1,300 polypeptides can be detected in wheat endosperm (Skylas et al 2000) (Figure 6). Even this degree of heterogeneity is only about a quarter of the expected number of polypeptides, based on estimates of the expression of mRNA in the developing endosperm (Clarke et al 2000). An important aspect of proteome analysis is the characterization of the resolved proteins. Some hundreds were characterized by Skylas et al (2000), on the basis of their N-terminal sequences. These are the basis of the groupings circled in Figure 6. More recent methods of characterization have involved peptide fragment mapping followed by mass spectrometry (reviewed by Skylas et al 2005).

NOMENCLATURE OF GLIADIN AND GLUTENIN PROTEINS

Effective communication in any branch of science requires an agreed language (“nomenclature”) to describe the many objects of attention. So, with gluten chemistry, we need to have an agreed set of names and meanings for the various protein components. The terms “gliadin” and “glutenin” are most obviously defined by the fractional extraction method of Osborne, but a century of active research provides justification of refining the original basis of the definitions, especially now that we are aware of the great heterogeneity of the gluten complex, plus the added knowledge of the genetics of the gluten proteins. Nevertheless, the first step in fractionation involves extraction into solution, so it is common practice to extract the water-soluble proteins from flour with dilute salt solution, followed by a solvent suited to the gliadins. This may be the traditional 70% aqueous ethanol, but it is more likely to be hydrogen-bond-breaking reagents (such as other alcohols, urea, ethylene glycol, formamide) leaving the glutenin protein in the residue.

An attempt to achieve agreement on gluten nomenclature was undertaken at the Sixth International Gluten Workshop (Wrigley et al 1996). The following definitions, adapted and up-dated from this publication, are divided between concept and practice, and the proteins are considered firstly as the “native” proteins and then as the individual polypeptides that are released after the rupture of disulfide bonds.

Gluten proteins (native)

Conceptually, gluten proteins are those that impart unique viscoelastic properties of dough. In practice, the gluten proteins are those contained in the mass remaining when dough is thoroughly washed under running water. The term “gluten” generally refers to the relevant proteins from wheat grain. However, on occasions, the term is (mis)used in relation to other crop species (e.g. “corn-gluten”). If there is any possibility of this confusion arising, the term “wheat gluten” should be used. There is also the distinct nutritional concept of “gluten” referring to the range of proteins that cause various food intolerances, especially celiac disease (see Chapter 13). In this

case, the term “gluten” is used in this context to cover the corresponding proteins from rye, triticale, barley and possibly oats.

Conceptually, gliadin proteins are the gluten proteins that exist in an extract of flour as monomeric polypeptides, with virtually all disulfide bonds being intra-polypeptide (see Chapters 2-4). On the other hand, the glutenin proteins are polymeric, having disulfide bonds joining between individual polypeptides of glutenin (see Chapters 5-7). There is the further conceptual distinction that the genes coding for the gliadin and glutenin proteins have specific locations in the wheat genome, as described below for the relevant polypeptides. In practice, this distinction means that gliadins have molecular sizes smaller than glutenin proteins, the dividing line between the two groups of proteins being an “apparent molecular weight” of about 100,000 Daltons. Thus, a practical separation of gliadin from glutenin in solution can be achieved by any method that separates proteins according to size, e.g. gel filtration, size-based gel electrophoresis, size-exclusion high-performance liquid chromatography (SE-HPLC), field-flow fractionation (FFF), and size-based capillary electrophoresis.

Gliadin polypeptides

Conceptually, there is the distinction that the gliadin polypeptides occur in groups (“blocks”), based on each of the several sets of tightly linked genes coding for the gliadin polypeptides. The main blocks of gliadin genes are located on the short arms of Group-1 and Group-6 chromosomes (referred to as the *Gli-1* and *Gli-2* loci, respectively) for all three wheat genomes (A, B and D). Gene location provides a conceptual distinction between the polypeptides of gliadin and glutenin, and also a complementary means of naming individual gliadin polypeptides, by reference to the specific alleles at these loci (e.g. referred to as *Gli-A1a*, *Gli-A1b*, etc.). When the gliadin polypeptides are fractionated (e.g., by gel electrophoresis or RP-HPLC), the pattern is made up of over-lapping combinations of polypeptides derived from each of the six blocks of gliadin genes. An emerging level of nomenclature is based on amino-acid (or nucleotide) sequence.

In practice, the gliadin proteins have been grouped according to their electrophoretic mobilities in polyacrylamide gel, cathodically at pH 3, with regions of mobility designated as α/β , γ and ω (Figure 4). An allele-based nomenclature (Metakovsky 1991) has been generally adopted, as described in Chapters 2-4. Because there are so many gliadin bands, it is advisable to use gliadin extracts from flour of standard genotypes as reference markers of mobilities for the gene-block nomenclature (in the form *Gli-A1a*).

The gliadin polypeptides may also be identified from SDS-PAGE patterns, but they are not as clearly resolved by size-based methods as by A-PAGE. Furthermore, in SDS-PAGE patterns, most of the gliadins share a mobility region with many glutenin subunits, unless there has been pre-fractionation to isolate the gliadins. However, fractionation of the ω -gliadins presents a special case, their identification being facilitated by their pre-

sence alone in the “D-zone” of an SDS-electrophoresis gel (Figure 5) (Khelifi et al 1992).

The HMW polypeptides of glutenin

Conceptually, the nomenclature of the HMW polypeptides of glutenin was originally based on their apparent sizes (after reduction of disulfide bonds) as indicated by SDS gel electrophoresis, in addition to the complementary evidence provided by the locations of genes coding for them on the long arms of Group-1 chromosomes (at the *Glu-1* loci) (see Chapter 5). The grouping of the HMW-glutenin polypeptides as those with largest size distinguishes them from the LMW-glutenin subunits. The location of the respective genes in the wheat genome also provides a good distinction between HMW and LMW subunits.

In practice, the naming of the HMW subunits has depended on their mobilities on SDS-gel electrophoresis. Payne and Lawrence (1983) specified numbers for many of the HMW subunits, together with some appropriate reference genotypes. These numbers for the polypeptide bands on the gel have in turn been linked to specific alleles, using a lower-case letter, e.g., *Glu-D1a* for the pair of HMW subunits numbered 2 and 12. Each locus comprises two tightly-linked genes, namely, an x-type (that encodes a larger subunit) and a y-type (that encodes a smaller-sized subunit). The individual subunits are thus designated according to the combination of locus and “x” or “y” type, e.g., subunits 5 and 10, coded by the *Glu-D1d* allele, are indicated as the “Dx5” and “Dy10” subunits.

The LMW polypeptides of glutenin

The concept of nomenclature for the LMW polypeptides of glutenin is similar to that for the HMW subunits, namely, that the LMW subunits are those of smaller apparent size on SDS-gel electrophoresis, and that the alleles (at the *Glu-3* locus) can be designated for the LMW subunits according to the respective loci on the short arms (in the case of the LMW subunits) of Group-1 chromosomes (e.g. as *Glu-A3a*) (see Chapter 6). In practice, no system of numbering for individual LMW subunits has been adopted, largely because of their greater complexity, compared to the HMW subunits.

Nevertheless, regions of mobility for the LMW subunits in an SDS gel have been divided into B and C (Figure 5). Because of the lack of numbers to specify LMW bands, greater reliance must be placed on the designation of the genetic allele, in the way recommended for the gliadin polypeptides (e.g., as *Glu-A3a*), using standard genotypes for reference purposes (Gupta and Shepherd 1990). The alleles for the LMW glutenin subunits can also be specified by indicating the allele for the (tightly linked) gliadin proteins on appropriate *Gli-1* loci (Gupta et al 1993b; Jackson et al 1996).

Ideally, nomenclature should be based on information from amino-acid and nucleotide sequences, and this type of data is now becoming available to permit a systematic application. There is growing evidence of

similarity groupings within the LMW polypeptides of glutenin, based on sequence studies, and also of equivalences between sub-groupings of the subunits compared to gliadin polypeptides, e.g., SHIPGLEK/R..., METSC/HIPG..., VRVPUPQL... . This as a potentially valuable “handle” on nomenclature.

BALANCING DOUGH PROPERTIES

One of the ultimate tasks of cereal science is to improve the “quality” of wheat through a better understanding of its relationship to the chemical composition of wheat flour. Selecting from the numerous possible aspects of describing the complex term, “quality”, we define it in relation to how suitable a sample is for producing a good end-product. The “quality” of a given sample is therefore specific to the end-product; its “value” is different depending on the potential use. For example, the quality of an excellent bread-making wheat may be poor in relation to noodle production, and even worse for cake manufacture. For most uses of wheat, dough properties are critical aspects of “quality”, it is the storage proteins of the wheat endosperm that are the main determinants of dough properties, such as dough strength, extensibility, dough stability. Additional factors may include attributes related to starch quality, such as pasting viscosity.

Dough properties have been characterized using laboratory equipments developed more than 70 years ago by applying principals designed to mimic industrial procedures (e.g., Swanson and Working 1933). These empirical procedures have served the wheat industry for selecting new wheat varieties, for characterizing trade samples, and as tools for quality control and for formulation (Walker et al 1997). In the last 15 years, computing technologies have fulfilled the needs of making dough-testing procedures more objective, more accurate and precise, with larger throughput, and with significantly less flour sample. These developments have involved online computerization of traditional methodologies, and small-scale (even micro-scale) versions of traditional equipment such as the Mixograph (Rath et al 1990), the Extensograph (Rath et al 1995) and the Farinograph (Haraszi et al 2004). One of the ways to improve the relationships between “quality”, based on end-product suitability and dough properties, is to involve fundamental rheology measurements in the characterization of doughs (Keentok 2002; Uthayakumaran 2002). This attitude has been a further a trend in the past decade. Another approach has involved the realization that high-resolution data from traditional dough testing contains huge amounts of previously unused information about the rheological properties of the dough (Anderssen et al 2004; Gras et al 2000).

Individual dough-property parameters describe only certain essential elements of dough properties. Depending on the final product, different levels of these attributes are required to get superior processing quality. For example, the balance of dough strength and extensibility are believed to be the most important factors governing the suitability of a flour to make good

bread (Bushuk and Békés 2002). However, for different types of breads, and even for different type of processing technologies, a diversity of dough-strength and extensibility values may provide the optimum balances needed in each case (Oliver and Allen 1992). For most traditional uses, wheat quality derives mainly from two interrelated characteristics: grain hardness and protein content with each end-use requiring a particular type of “protein quality”. These aspects of quality are mainly determined by the protein molecular structure which, in turn, controls the interactions of the proteins during the bread-making process (Bushuk 1998; Shewry et al 1999).

THE PROTEIN BALANCE: GLIADIN-GLUTENIN AND MORE

The polypeptide composition of a wheat-flour sample is determined by genotype (“G” effects), due to the allelic composition of the three HMW subunits of glutenin, the three LMW subunits and six gliadin-coding loci. There is extensive polymorphism at certain loci, most of all at the *Glu-B1* and *Glu-B3* loci, and at each of the *Gli-1* loci. With many allele combinations possible, there is great potential for biodiversity. This qualitative aspect of protein composition is perturbed further by the expression levels of genes determining the absolute and relative amounts of different gene products. The effects of growing conditions on the expression levels (“E” effects of growth environment) and the different sensitivities of the expression levels on the individual genes (“G x E” effects) provide the basis for even wider variation in protein composition.

The picture of the qualitative and quantitative aspects of variation in protein composition is further colored by the actions of unknown numbers of genes involved in the deposition of the storage proteins. One of the most important aspects of this process is polymer formation from the glutenin subunits. The size distribution of the polymeric glutenin (a structural feature directly related to functional properties; see Chapter 7) is determined by genetic, environmental and G x E factors.

The range of protein-balance factors

The complexity of relating protein composition to quality derives from the fact that the question can (and has to be) investigated on different levels of protein composition, namely, protein content, the ratio of polymeric to monomeric protein, the ratio of HMW to LMW glutenin subunits, and the proportions of x- and y-type HMW glutenin subunits. These various parameters can be determined for a specific flour sample to see if there is a “good balance” between the various components in the sample, thereby to satisfy quality-related criteria. The polymeric glutenin is mostly responsible for the elasticity of the dough, whereas the monomeric gliadins are the extensibility-related characters in the system (Hoseney, 1986). Thus, the ratio of polymeric to monomeric proteins (the glutenin-to-gliadin ratio) can be directly related to the balance of dough strength and extensibility of the sample.

To investigate these relationships, there are important requirements for the experiments to be considered to be valid. Because dough properties are significantly dependent of protein content, the balance of glutenin-to-gliadin ratio can best be compared among samples with similar protein contents. Meanwhile, the composition of both the glutenin and gliadin proteins has to be taken into account because, for example, at the same glutenin-to-gliadin ratio, the balance of HMW-to-LMW glutenin subunits in the polymeric fraction can significantly alter dough strength and extensibility. Clear examples have been reported to demonstrate the extremes in dough properties that result from drastic changes in HMW-to-LMW ratio. For example, dough strength systematically decreased, while extensibility increased, as a result of decreasing the HMW-to-LMW subunit ratio for sibling lines of an Olympic x Gabo cross (Lawrence et al 1988), providing single-, double- and triple-null lines for HMW subunit-coding genes (Uthayakumaran et al 2001; Beasley et al 2002). On the other hand, the increased number of copies of the genes coding for Dx5 subunits in transgenic wheat samples resulted in doughs so strong that it was not possible to mix them with traditional equipment (see Chapter 12).

Further manipulation of protein balance in dough can be achieved by systematically changing the ratio of x- to y-HMW glutenin subunits (Butow et al 2003b), whilst maintaining equivalent levels for protein content, glutenin-to-gliadin ratio and HMW-to-LMW subunit ratio. The presence and the relative levels of individual polypeptides can also be related to quality attributes. For example, the “imbalance” in glutenin-subunit composition caused by the over-expression of subunit Bx7 in certain wheat varieties around the world (Glenlea, Red River, Bankuti 1201, Chara, Kukri) provides extra dough strength and better overall bread-making quality, compared to samples with comparable protein content and glutenin-to-gliadin ratios (Butow et al 2003a; Juhasz et al 2003). The effects, due to genotype, growth environment and G x E interactions, alter each of these balances simultaneously, so complex methodologies are required to separate and evaluate the effects on quality at the various structural levels. Nevertheless, greater understanding of these factors offers the potential to predict grain quality with respect to dough-forming potential (see Chapters 8 and 9).

Experimental approaches

The effects due to genotype, growth environment and G x E alter each of these balances simultaneously, so complex methodologies are required to separate and evaluate the effects on quality at the various structural levels.

Much of our knowledge about the effects of specific proteins on the functional properties of wheat flour is based on correlative studies. For this purpose, the functional characteristics of every member of a population of samples are determined. The population is selected to have variation at a number of alleles, and thus provide a range in protein composition. The re-

relationships between the differences in quality and the differences in genetic makeup (and thus qualitative differences in protein composition) are established using statistical methods. A major limitation of this approach is that the statistical evaluation is carried out on populations where the effects of several compositional variations (such as protein content, protein composition and protein-size distribution) are superimposed on each other (Békés et al 2004). Even with quite large sample sets, variations caused by the usual experimental errors can easily conspire to produce conflicting results in different sample populations, unless the effects of specific differences in protein composition are relatively large.

The classical technique of reconstitution provides insight into the effects of flour components on flour quality by directly altering the chemical composition of the flour. Using this approach, the main components of flour are isolated and recombined in various ways for direct measurement of the properties of the reconstituted flour samples. The results of many reconstitution studies have shown that the intrinsic differences between wheat cultivars could, at least in part, be related to their protein components. Two aspects of the role of protein which were identified were the amount of protein present and its source (the cultivar from which the protein was isolated). In today's terms, we would deduce that this implied differences between the proteins from different cultivars (Harris and Sibbitt 1942; Finney 1943). It is just such differences that can be shown by modern biochemical techniques.

A variation of the above reconstitution technique is to vary the amount of a specific flour component in a systematic way. The now widely-accepted relationships between the molecular-weight distribution of gluten proteins and dough properties was first explored systematically by separating the gluten into a range of fractions of different molecular-weight distribution, followed by mixing studies of "reconstituted" flours made by mixing the isolated glutenin fractions with the other flour components (MacRitchie 1987).

Using these approaches, the effects of protein content, protein composition and protein size distribution have all been determined separately. Three major aspects of protein composition (protein content, glutenin-to-gliadin ratio and HMW-to-LMW GS ratio) have been determined separately in the same sample set, providing a comparison of the relative importance of the different roles that each aspect of protein composition plays in determining the various dough- and bread-quality parameters (Uthayakumaran et al 1999, 2000, 2001). Such studies play an important part in determining the functional role of each aspect of protein composition.

Direct testing of specific proteins

More objective assessment of the experimental variables has been possible with the development of very small-scale dough-testing equipment

and methodology, permitting the analysis of significantly smaller samples with better reproducibility and absence of operator bias. This has facilitated a wide range of research in which only limited amounts of test material have been available, as well as providing more objective, precise assessment of data (Békés et al 2003; Békés and Gras 1999).

Even using small-scale dough-testing equipment, the major difficulty for direct, *in vitro* functional studies is the isolation of sufficient amounts of pure proteins. Modern biochemical technology has provided an alternative approach, where the isolated gene for the desired wheat protein is inserted into a foreign genome, such as *Escherichia coli*, yeast or insect-cell lines. This technique, called “heterologous expression”, allows the production of single polypeptides in relatively large quantities, thereby facilitating protein purification. A further advantage of the technique is the ability to produce novel proteins, using genetic engineering to alter the original gene before insertion into the foreign genome. This permits the determination of the role of structural features, such as the number and location of cysteine residues of a polypeptide in determining functional parameters.

The effects of gliadin on dough-mixing properties have been determined by the reconstitution method (MacRitchie 1987). Addition of isolated gliadin or specific gliadin classes to flour results in a weaker and less stable dough, as shown by decreases in mixing time and maximum resistance and an increase in resistance breakdown (Uthayakumaran et al 2001; Hussain and Lukow 1997). Such doughs exhibit increased extensibility and poorer baking performance. In every case, the addition of lower-molecular-weight proteins effectively reduces the average molecular weight of the protein in the composite flour. The extent of these changes varies among the gliadin classes (α -, β -, γ - and ω -gliadins), with the ω -gliadins having the largest effect on dough-mixing time (Fido et al 1997; Uthayakumaran et al 2001; Murray et al 1998).

Study of the monomeric-to-polymeric balance

Apart from the amount of protein in flour, probably the most important characteristic of gluten that determines the mixing time of dough is the size distribution of the gluten proteins. This consideration involves the ratio of the monomeric-to-polymeric proteins, and specially the size distribution of the polymeric proteins. The glutenin polymers are formed from the glutenin subunits by the formation of disulfide bonds. The weakening effects of reductants on dough properties are caused by the rupture of these disulfide bonds, with consequent reduction in average molecular weight, and consequent reduction in the time for the dough to mix to peak resistance.

Conversely, oxidants lead to the formation of more inter-chain disulfide bonds, increasing the average molecular weight of the proteins and strengthening the dough. The measurement of the size distribution of these polymers is one of the outstanding problems faced by cereal chemists in the

near future. The addition of monomeric glutenin subunits to a base flour reduces the average molecular weight of the protein in the composite flour. In such an *in-vitro* experiment, the addition of glutenin subunits would not be expected to have the effect on dough properties that would be expected for an *in-vivo* experiment, because the subunits would not form part of the extended disulfide-linked glutenin structure. Meaningful estimates of the effects of added glutenin subunits on dough properties could be made only if they could be chemically incorporated into the glutenin polymer, as they would be in an *in-vivo* experiment.

To perform this step of chemical incorporation, some assumptions had to be made, since the polymer structure is still not well understood, despite recent significant strides in elucidating glutenin structure (Keck et al 1995; Shewry and Tatham 1997). Presuming that molecular-weight distribution is an important determinant of dough properties, and given the known effects of oxidants and reductants on dough properties, it should be possible to partially reduce the glutenin and subsequently re-oxidize it to approximately the same molecular weight distribution without significant changes to its eventual functionality. It may not be expected that the structure of the polymer would be exactly the same after reduction and oxidation, but its molecular weight distribution should be essentially recovered.

Studies of the effects of a range of reductants and oxidants on the functionality of gluten proteins during dough mixing showed that it was possible to effectively destroy dough functionality with a reductant, and then to recover functionality by subsequent oxidation (Békés et al 1994b). Although several reductants were tested, dithiothreitol was found to be the only one of these whose action on dough-mixing properties could be readily reversed. Careful selection of the oxidant, its concentration and reaction conditions allowed essentially complete recovery of the original dough-mixing properties. For the oxidation step, bromate was the oxidant of choice, performing better than iodate, permanganate or hydrogen peroxide. The parameters of this reduction/oxidation procedure have been optimized so that there is less than 5% difference in mixing time, peak dough resistance and dough stability (resistance breakdown) between a treated and untreated flour. Under these conditions, no significant difference in the size distribution of the proteins isolated from the two samples could be detected (Békés et al 1994b).

This reduction/oxidation procedure (“incorporation”) has since been applied to incorporate a wide range of partially purified fractions or individual purified glutenin subunits into the polymeric phase that we refer to as “glutenin” (Békés et al 1994a, 1994c; Sapirstein and Fu 1996; Veraverbeke et al 1999). The reduction-oxidation procedure has been shown not to alter mixing properties of mixtures of flour and isolated gliadin (Murray et al 1998). Thus, the presence of intra-molecular disulfide bonds in gliadin does not seem to interfere with the reduction-oxidation of glutenin.

Polymer-chemistry considerations

Although the effect of the glutenin on wheat quality has largely been considered in relation to subunit composition, there is the added need to introduce concepts of polymer chemistry (Chapter 7), acknowledging the interactions that occur with the wider range of components of dough. Polymer science indicates the importance of the size distribution for such molecules as a critical principle governing the physical properties of synthetic polymers (MacRitchie 1992; Weegels et al 1996a, 1996b). For example, molecules below a certain size limit (threshold level) do not contribute to the strength properties of a polymer composite. By analogy, size distribution should be important for the gluten proteins (Southan and MacRitchie 1999).

The significant relationship between the amount of polymeric gluten proteins present in the flour and dough strength is well established (Orth and Bushuk 1972; Dachkievitch and Autran 1989; Gupta et al 1993a; Weegels et al 1996a; Butow et al 2003a, 2003b). Gluten proteins have two levels of aggregation before starting to form the gluten polymer. At the first level HMW- and LMW-subunits of glutenin form covalent polymers and on the second level, larger aggregates, called “glutenin macropolymers” (GMP) (Graveland et al 1982; Weegels et al 1996b) or “unextractable polymeric proteins” (UPP) (Gupta et al 1993a), are formed and stabilized by hydrogen and disulfide bonds. The level of this second aggregation is highly influenced by allelic composition (Hamer and van Vliet 2000; Rhazi et al 2003).

Methodologically, the determination of %UPP (Figure 3) is based on a two-step extraction procedure, firstly without and then with sonication, followed by a SE-HPLC separation of polymeric and monomeric proteins (Gupta et al 1993a). The role of sonication is to make the originally unextractable polymers extractable. Not the size, but the amounts of the non-sonicated and then the sonicated extracts are used for the calculation of the amount of unextractable polymeric proteins, as the percentage of total polymeric protein content. Therefore, the %UPP is a very simple way to characterize the overall size distribution of the polymeric proteins in the original sample, without creating artefacts caused by the sonication.

During mixing, the size of protein aggregates decreases (Tsen 1967; Mecham et al 1965). These changes involve both the amount and the average molecular weight of the glutenin macropolymer (Skerritt et al 1999a, 1999b; Weegels et al 1997; Kuktaite et al 2004), reaching its minimum in doughs mixed to peak resistance. The remaining macropolymer contains a reduced amount of HMW-glutenin subunits (Bushuk et al 1997; Skerritt et al 1999b). These events occur due to changes in chemical and physical bonds in the protein polymer and aggregates. Only a small proportion of the thiol and disulfide groups are rheologically active (Bloksma 1975). The rupture of disulfide bonds to form exposed thiol

grouéps was found during pin mixing, but not in the sheeting process; apparently, disulfide-bond rupture is thus not required for dough development, although the rupture and reformation of hydrogen and hydrophobic bonds are known to play significant roles in dough formation (Sutton et al 2003).

CONCLUSION

Conversion of wheat flour into bread involves an optimum combination of ingredients and processes. Of the ingredients, the key one is the flour. Of the flour constituents, it is the protein that gives the flour, when mixed with water, the ability to form a viscoelastic dough, which in turn converts into an attractive and nutritious loaf of bread after baking. Similar considerations also apply to the many other food products that rely on the unique rheological properties of wheat flour. This ability is endowed by the complementary gluten-protein fractions, gliadin and glutenin. Optimum processing quality requires a unique balance between these two types of protein. This book presents the current state of scientific knowledge of the factors that contribute to this balance, thereby providing a practical basis for devising better testing methods for grain, for improving the genotypes available, and for understanding consumer issues (see Chapters 8-13).

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