BIOCHEMICAL PROPERTIES OF WHEAT GLUTEN PROTEINS IN RELATION TO BREADMAKING QUALITY

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BIOCHEMICAL PROPERTIES OF WHEAT GLUTEN PROTEINS IN RELATION TO BREADMAKING QUALITY

BY

BIN XIAO FU

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ABSTRACT

Fu, Bin Xiao, Ph.D., The University of Manitoba, November 1996

Biochemical Properties of Wheat Gluten Proteins in Relation to Breadmaking Quality

Advisor: Dr. H.D. Sapirstein

The unique ability of wheat flour to produce leavened bread is due primarily to physicochemical uniqueness of the gluten proteins, gliadin and glutenin. This study was undertaken to gain new knowledge on the chemistry and functionality of these proteins to further elucidate the relationship between protein composition/structure and functionality in the breadmaking process.

At the outset, a new method was developed for fractionation of flour proteins, based on different aqueous solutions of 1-propanol. The fractionation procedure yielded four relatively distinct protein fractions, one monomeric protein fraction (mainly gliadins), and three subfractions of polymeric protein (mainly glutenins). The latter comprised 50% 1-propanol soluble glutenin (precipitated by 70% 1-propanol), 50% 1-propanol insoluble glutenin (soluble in 50% 1-propanol plus reducing agent), and residue proteins (insoluble in 50% 1-propanol plus reducing agent).

The fractionation procedure was used to compare the protein distribution of flours of seven Canadian wheat cultivars of diverse breadmaking quality. There was relatively little variation among the seven cultivars in the proportion of total flour protein comprising monomeric (48-52%) and residue (14-18%) proteins. The soluble- and insoluble-glutenin fractions showed much greater inter-cultivar variation, at 10-20% and 12-28% of total flour protein, respectively. The variation in these two glutenin fractions

explained 80-90% of the variation in dough strength parameters, and 70-80% of the variation in loaf volume. It is noteworthy that the flour sample with the highest ratio of insoluble to soluble glutenin, as well as marginally the lowest concentration of monomeric proteins was Glenlea which possesses very strong dough mixing characteristics.

A comparative analysis of the quantitative and qualitative subunit compositions of the soluble- and insoluble-glutenin fractions was subsequently determined by reversed phase-high performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All seven wheat cultivars showed no qualitative differences in subunit composition of the two glutenin fractions. Also, there was no significant variation between soluble- and insoluble-glutenin in the relative amounts of total high molecular weight (HMW) or low molecular weight (LMW) subunits. However, significant variation was observed in the relative amounts of certain HMW- and LMW-subunits. Most notably, insoluble glutenin contained a greater proportion of *Glu-1Dx* subunits, especially *Glu-1Dx* 5, and a lesser proportion of *Glu-1Bx* subunits than soluble glutenin. This result indicated that the *Glu-1Dx* subunits are most important to the formation of the more insoluble glutenin (polymers of larger molecular size), which in turn, appears to be related to dough strength.

A fractionation and fortification study was then made to directly determine the effects on dough mixing of gliadin- and glutenin-enriched protein fractions added to a strong and weak base flour, respectively. To this end, gliadin- and glutenin-rich fractions from three flours with diverse dough strength were carefully isolated from 0.5 M NaCl insoluble flour residues by repeated extraction with water. There was no intercultivar

variation in the dough weakening effect caused by addition of the gliadin-rich fractions. In contrast, the dough strengthening effect of the glutenin-rich fractions was cultivar dependent, it was directly related to the strength of the flour used to isolate the fraction, e.g. the fraction from the strongest wheat (Glenlea) had the greatest effect.

To further identify the nature of the intercultivar variation in glutenin functionality, total HMW- and LMW-glutenin subunit fractions were isolated from flour samples of four wheats of different strength. Subsequently, these fractions were chemically incorporated, at a suitable concentration, into a base flour dough by reduction and oxidation. The results indicated that incorporation of LMW subunits from different wheat cultivars had marginal effects on the dough mixing properties of the base flour. In contrast, incorporation of HMW subunits produced a significant and substantial increase in dough strength, however, no qualitative intercultivar differences in dough-strengthening effects were observed among the HMW subunit isolates.

In the final part of the thesis research, the solubility in distilled deionized water (DDW) of gluten prepared without and with added salt (NaCl) was investigated. It was found that gluten protein solubility in DDW increased with increasing salt concentration. The DDW solubilities of Katepwa gluten proteins prepared in DDW, 0.2% NaCl and 2% NaCl were 27, 52, and 85%, respectively, after four sequential extractions with DDW. It was apparent that the remarkable solubility effect resulted from a salt-induced conformational change in gluten proteins making them more hydrophillic. Additionally, it was found that gliadin in gluten of a stronger wheat cultivar was more readily extractable with DDW than gliadin in gluten of a weaker cultivar; the first DDW extraction of gluten prepared with 0.2% NaCl solubilized 57.6, 28.2 and 4.8% of

gliadins in glutens of Glenlea (very strong), Katepwa (strong) and Harus (weak), respectively. This result is attributed to stronger gliadin-glutenin interaction in weaker than in stronger glutens. This hypothesis would predict that cultivars with strong gliadin-glutenin interactions require less mixing for optimum dough development, and vice versa.

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Chapter 1

INTRODUCTION

Wheat is the leading cereal grain in terms of production and commerce and the world's most important food crop. Its importance arises from the unique ability of wheat flour to form a viscoelastic dough when mixed with water. The viscoelastic dough can retain gases produced by yeast fermentation, and upon heating is transformed into a light textured and tasty loaf of bread. Wheat flour doughs consist largely of starch granules surrounded by a hydrated film of gluten proteins. There is good evidence that the rheological properties of dough derive directly from analogous properties of wheat gluten proteins whose composition and structure is under genetic control. Accordingly, the utility of wheat flour for breadmaking mostly depends on the structure and properties of its gluten proteins.

Gluten proteins largely correspond to the wheat storage proteins (mainly gliadin and glutenin) in wheat endosperm which represent about 80% of total protein in a typical flour. Gliadins are single chain polypeptides (monomeric proteins) with either no or only intra-chain disulfide bonds, while glutenin occurs as polydisperse polymers of varying size with individual polypeptides (subunits) linked by interchain disulfide bonds. Wheat flour doughs possess a unique combination of two physical properties, viscosity and elasticity. Viscosity is generally associated with the gliadins, while elasticity is understood to be a function of glutenin. A precise combination of these properties is critical in determining the functional properties of doughs. As a result of the evidence

that there are major differences in the quality of gluten proteins of different wheat cultivars (Finney and Barmore 1948), much research has focused on the biochemical properties of gluten proteins and the molecular basis of those differences.

Studies on the relative proportion of gliadin or glutenin in total flour protein and the molecular weight distribution of glutenin have been an important part of the overall attempts to elucidate the relationship between protein structure and breadmaking quality. However, the biochemical interpretation of these studies has been hampered by cross-contamination of the two major protein classes and poor separation of glutenins of different sizes (MacRitchie 1992). It has long been realized that the distinction between Osborne (1907) solubility classes of wheat flour proteins for example is not sharp because of the overlapping solubility of proteins in a highly heterogenous mixture of polypeptides (Schofield and Booth 1983). In addition to the difficulty in solubilizing native glutenin, gel filtration and size-exclusion (SE)-HPLC lose resolution for proteins above a certain molecular size. This results in poor or no fractionation of glutenins of different molecular weights (Wahlund et al 1996). It would be a fair assessment that the "ideal" fractionation procedure, which has eluded cereal chemists since the time of Osborne (1907), may not exist.

A direct way to study the functional properties of flour proteins is by fractionation and reconstitution. In reconstitution experiments, flour protein fractions are separated and then either added in varying amounts to a base flour or interchanged with equivalent components of flours with contrasting properties. The fractions responsible for the intercultivar differences in dough mixing properties or baking quality can thus be

identified. However, conclusions based on results of various reconstitution studies are not in agreement. Apart from the possible changes in protein functionality during fractionation, conflicting conclusions have likely arisen from differences in the composition of the fractions prepared in different laboratories (Chakraborty and Khan 1988a, 1988b). Accordingly, for fractionation and reconstitution studies to be successful a fractionation procedure is required which provides a relatively clean separation of gliadin and glutenin under non-denaturing conditions.

In native glutenin, a large number of different subunits (approximately 20) are linked together by interpolypeptide disulfide bonds into gigantic polymeric molecules (Wrigley 1996). Excellent progress has been made on the subunit composition and breadmaking quality of glutenin (Payne 1987; Shewry et al 1992; Gupta et al 1995). However, our understanding of the functionality of glutenin subunits is mainly derived from statistical studies, which are valid for identifying marker proteins but do not necessarily indicate the cause-and-effect relationships. Recently, a technique for incorporating subunits into the chemical structure of glutenin by partial reduction, followed by oxidation has been developed by Bekes et al (1994a). This method can be now used for direct study of the functionality of specific glutenin subunit(s).

The unique properties of wheat flour are primarily a function of the behaviour of gluten proteins in an aqueous environment. Water is the intermediary for all types of interactions and reactions that occur during dough formation and development. Accordingly, the gluten-water relationship is of great practical importance in processing wheat into food products. However, most investigations on biochemical properties of

gluten proteins have involved the use of strong dissociating or reducing solvents. It is important to know the physicochemical behaviour of gluten proteins in water if we are to understand the molecular basis of their unique functionality in processes such as breadmaking.

In summary, the problem of relating composition and structure of flour proteins to functional properties in breadmaking is complicated by several factors: 1) the heterogeneity of the major protein fractions, 2) the difficulty of separating or isolating pure fractions, 3) the limited solubility of glutenin, 4) the labile nature of the native structure of the proteins in terms of their functionality, and 5) the likely interaction between the gliadin and glutenin during their deposition in wheat endosperm and during processing of flour into bread.

The broad goal of this thesis project was to extend the knowledge on the biochemical properties of wheat gluten proteins in relation to the breadmaking quality of a diverse set of Canadian wheats. The practical focus of the study was the elucidation, at the molecular level, of the basis of the extra strong dough properties of Glenlea wheat, a cultivar of the Canada Western Extra Strong Red Spring wheat class.

At the outset, a fractionation procedure was developed which would provide a clean separation of flour proteins into structurally distinct fractions (i.e., monomeric and polymeric proteins). Next, the composition of the proteins of flours milled from a group of wheat cultivars of diverse dough strength (see Appendix I) was determined by the developed procedure. Finally, the relationships between the quantity of various protein fractions and dough properties and breadmaking quality were determined.

A fractionation and fortification study was undertaken to determine the effects of specific protein(s) fractions on physical dough properties and the level of agreement with complementary data obtained by statistical methods in Chapter 4. To this end, gliadin- and glutenin-rich fractions were carefully isolated by a mild procedure to avoid denaturation. The fractions isolated from different wheats were added to strong and weaker base flours respectively, and their effects on dough mixing properties were determined using the mixograph.

Once the key role of glutenin proteins in governing varietal differences in breadmaking potential was confirmed, the next step was to determine the relative contribution of the high molecular weight (HMW-) and low molecular weight (LMW-) glutenin-subunit fractions to dough strength. Accordingly, the HMW- and LMW-glutenin subunit fractions were isolated from different cultivars and chemically incorporated into a base flour by reduction/oxidation, and their effects on dough mixing properties were evaluated.

Several other relevant experiments were included in the thesis project. In preliminary experiments on fractionation of gluten proteins, a large increase in gluten protein solubility in water was found when the gluten was prepared in the presence of salt; the higher the salt concentration, the higher the gluten protein solubility. Studies were therefore undertaken to determine the quantity and composition of water-extractable proteins from glutens prepared using water and salt solutions of two concentrations (0.2% and 2% NaCl). The disaggregation/solubilization behaviour in water of glutens isolated from different wheat cultivars was compared in an attempt to obtain new information to

explain intercultivar differences in dough mixing requirements.

The results of the thesis research are presented herein as a series of seven chapters. Two articles have already been published (Fu and Sapirstein 1996, Fu et al 1996) based on Chapters 3 and 8, respectively. Each chapter is written in the style of a scientific article. To make the thesis fully comprehensive, chapters covering overall introduction, literature review, general discussion and summary of contributions to knowledge are included. The flow of information in the thesis is as follows:

Chapter 1 is a general introduction which presents the rationale for the study.

Chapter 2 gives general review of the pertinent literature.

Chapter 3 describes a new method that was developed for fractionation of monomeric and polymeric wheat proteins based on their differential solubility in different aqueous solutions of 1-propanol.

Chapter 4 examines the composition of the flour proteins of seven wheat cultivars of diverse dough strength using the fractionation procedure reported in Chapter 3. Also, this chapter covers the relationships between the concentration of each protein fraction in flour and physical dough properties and breadmaking quality.

Chapter 5 compares quantitative and qualitative subunit compositions of 50% 1-propanol-soluble and -insoluble fractions of glutenin, both of which were shown in Chapter 4 to be closely related to breadmaking quality.

Chapter 6 deals with the application of a unique gluten protein fractionation approach (resulting in gliadin- and glutenin-rich fractions). Fortification experiments in a mixograph were performed to measure the direct effects of these fractions from

different flours on physical dough properties.

Chapter 7 continues the theme explored in Chapter 6. It reports the results of experiments on the effect of total HMW- and LMW-subunit fractions of glutenin from different wheat cultivars on dough mixing properties using a chemical incorporation procedure. The objective of these experiments were to determine if intercultivar variation in dough mixing strength depends on the type and/or the amount of these two fractions.

Chapter 8 provides novel evidence on the solubility of gluten proteins in water when the gluten was originally prepared in the presence of salt.

Chapter 9 compares the disaggregation/solubilization behaviour in water of glutens prepared from different wheat cultivars according to the procedure developed in Chapter 8.

Chapter 10 is a general discussion of the results. The chapter also discusses the implication of the results in explaining intercultivar differences in breadmaking potential of wheat flour.

Chapter 11 is a summary of contributions to knowledge resulting from the thesis research.

Chapter 12 is the list of references cited in the thesis.

Chapter 2

LITERATURE REVIEW

Introduction

Many cereal chemists have devoted considerable effort to the search for the fundamental nature of protein quality for breadmaking since the demonstration of the relationship between the breadmaking quality of wheat flour and its protein quantity and quality (Finney and Barmore 1948). Enormous advances in knowledge of the functionality of wheat proteins have been made over the last two decades in particular, with the application of modern biochemical and biophysical methods frequently focused on individual polypeptides. Needless to say, there remains much to be done to fully understand the molecular basis of the functionality of wheat proteins in relation to breadmaking quality.

The present thesis project encompasses several aspects in the elucidation of the relationship between the structure and functionality of wheat proteins. Accordingly, this review will cover in detail aspects such as composition and structure of gluten proteins, fractionation of flour proteins and relationships to mixing and baking properties, and reconstitution/fortification studies to assess the functionality of gluten proteins. Documentation is scarce in the literature on the solubility of gluten proteins in water and the interactions between gliadin and glutenin, and therefore will not be included in this review. However, the few research articles available dealing with the latter areas are referenced in the relevant chapters of this thesis.

Composition, Structure, and Genetics of Wheat Gluten Proteins

Wheat proteins comprise an extremely heterogenous mixture of molecular species with different properties and functions in the wheat grain. The major wheat endosperm storage proteins, the gluten proteins, are considered essential for breadmaking quality because they confer the viscoelasticity on doughs. The storage proteins account for about 80% of the total amount of flour proteins, with the gliadin and glutenin each accounting for about 40%. The gliadins are single chain polypeptides, i.e. monomeric proteins with either no or only intra-chain disulfide bonds, In contrast, the glutenins exist as polymers of varying size in which the individual polypeptides (subunits) are linked by interchain disulfide bonds.

The gliadins have been further classified into four groups as α-, β-, γ-, and ω-gliadins, based on their mobilities in low pH gel electrophoresis (Woychik et al 1961, Bushuk and Sapirstein 1991). α-, β-, and γ-gliadins (relative molecular mass Mr = 32,000-44,000) are relatively similar proteins but distinctly different from the ω-gliadins (Mr = 50,000-70,000). The distinct feature of the latter group is that they are normally completely deficient in sulfur-containing amino acids and have different secondary structure (Tatham et al 1990). A new classification of the gliadins into sulfur-rich (α-, β-, γ-) and sulfur-poor (ω-) fractions has been proposed (Shewry et al 1986). Upon synthesis, gliadin proteins probably fold rapidly into conformations that permit pairs of cysteine residues to come into close association, whereupon they are oxidized to disulfide bonds (Kasarda 1989). By two-dimensional electrophoresis techniques, at least 50 different gliadin polypeptides have been identified in a single wheat cultivar (Wrigley

1970). The highly polymorphic nature of the gliadins is a unique genotypic trait of wheat which has been widely exploited for identification of wheat genotypes.

Glutenins occur as very large polymers, their true molecular weights have not been determined. Indirect evidence suggests that these proteins are highly polydisperse with an average molecular weight well above 1 million (Meredith and Wren 1966, Payne and Corfield 1979). A wheat genotype may contain as many as 20 subunits after complete reduction of the disulfide bonds of the polymer (Payne and Corfield 1979). As with gliadin polypeptides, allelic variation occurs in glutenin subunits. Glutenin subunits are classified into two main types, based on their electrophoretic mobilities in SDS-PAGE under fully reduced conditions. The group of faster moving subunits, with *Mr* of 30,000-55,000, are considered the low molecular weight (LMW) -glutenin subunits (GS), while the group of slower moving subunits, with *Mr* of 95,000-140,000, are considered the high molecular weight (HMW)-GS. HMW subunits have also been called "A" subunits, while LMW-GS have been further subdivided into "B" and "C" group subunits on the basis of their SDS-PAGE mobilities, with *Mr* of 40,000-51,000, and 31,000-36,500, respectively (Payne and Corfield 1979). An additional group of LMW-GS, with mobilities between HMW and B group LMW subunits, are called the D glutenin subunits (Jackson et al 1983), presumably because they were discovered after assignment of the A, B, and C designations. More recently, the so-called D-glutenin subunits have been identified as mutated ω-gliadins containing one cysteine residue (Masci et al 1993).

Both gliadin and glutenin are very rich in the amino acids glutamine and proline, with combined proportions ranging from about 70 mol% in the ω-gliadins to about 45

mol% in the HMW subunits. HMW-GS are also rich in glycine (about 20 mol%) and ω-gliadins are rich in phenylalanine (about 9 mol%) (Shewry and Tatham 1990). These characteristic amino acid compositions derive from one particular structural feature of the proteins: the presence of repeated amino acid sequences. The latter are based on one or more short peptide motifs ranging in length from three to nine residues. Repeated sequences of amino acids account for 30-50% for S-rich gliadins and LMW-GS, about 70% for HMW-GS, and close to 98% of the ω-gliadins (Tatham et al 1990). The repetitive domains with unusual secondary structures appear to be partly responsible for the unique properties of gluten proteins (Kreis and Shewry 1989).

In the case of HMW-GS (Shewry et al 1992), the repeats are present in a central domain which varies in length from about 480 to 680 residues. This is flanked by shorter non-repetitive domains at the N-terminus (81 to 140 residues) and C-terminus (42 residues), which are rich in α-helical structures. Molecular sizes of the subunits varies mainly according to the extent of the repeated sequences. Circular dichroism spectroscopy (Tatham et al 1984) and intrinsic viscosity studies (Field et al 1987) indicated that these repeats formed β-turns with an extended rod like structure. It was proposed that the β-turns were regularly organised to form a loose β-spiral structure, and this was confirmed by scanning tunnelling microscopy imaging (Miles et al 1991) which showed that the HMW subunits do indeed have a rod-like structure with a diagonal periodic banding pattern possibly corresponding to the β-turns in the spiral.

Similar studies of the S-poor C hordeins of barley, which are homologous to ω-gliadins, have shown that their repetitive domains also form a loose spiral structure

(Tatham et al 1985; Field et al 1986). This structure contains short sections of poly-L-proline II as well as β-turns, giving the whole protein a stiff, worm-like structure (I'Anson et al 1992). It was proposed that as the protein-protein interactions increased, the structure might change due to extensive hydrogen bonding, with a decrease in β-turns and an increase in intermolecular β-sheet structure conformations (Tatham and Shewry 1995).

The S-rich gliadins and LMW-GS are similar in terms of their secondary structures (Tatham et al 1987). Repeated sequences account for about 30 mol% to 50 mol% of these proteins, in contrast to the more extensive repeats in the S-poor gliadins and HMW glutenin subunits. They have a clear two domain structure. The N-terminal repetitive domains are rich in β-turns and may form a regular spiral structure, while the non-repetitive C-terminal domains are rich in α-helix and appear to be more compact (Thomson et al 1992).

All HMW-GS have a single cysteine residue in their C-terminal domains, and three or five cysteine residues in their N-terminal domains (Shewry et al 1989). This suggests the importance of these subunits in relation to glutenin polymer formation via interchain disulfide bonds. The α-, β-, and γ-gliadins contain an even number of cysteine residues in their C-terminal domains which apparently form only intramolecular disulfide bonds. The LMW-GS (except the D-group) have a similar overall structure to the S-rich gliadins, but have a cysteine residue within the N-terminal domain (Okita et al 1985; Colot et al 1989; Tao and Kasarda 1989; Lew et al 1992). This is unlikely to form intramolecular disulfide bonds with cysteine residue in the C-terminal domain because

of the rigidity imposed by the repetitive sequence. In addition, LMW-GS have seven cysteine residues in their C-terminal domains; at least one of them will be unpaired.

Although knowledge is accumulating on the sequence and structure of glutenin subunits, questions about how they are assembled in the large polymeric molecules are yet to be resolved. To elucidate the composition/structure/functionality relationships for glutenin in breadmaking, we need knowledge about the polymeric structure of glutenin for it is this level of structure that is functionally involved in breadmaking. A major challenge to understanding the polymeric structure of glutenin has been the complexity of the system and its limited solubility. The glutenin polymers range in *Mr* from about 1×10^5 to 10×10^6, and consist of 3, 4, or 5 HMW subunits, together with about 15 LMW subunits. Despite the lack of data on the precise details of the disulfide bonds in glutenin, several models have been proposed for the structure of polymeric glutenin (for review see Bushuk 1994).

HMW subunits are encoded by genes at the complex *Glu-1* loci located on the long arms of the group one chromosomes (1A, 1B and 1D) of hexaploid bread wheat. Each locus contains two tightly linked genes, one encoding a subunit of slightly higher *Mr* (80,000-88,000 as calculated from nucleotide sequences) and the other encoding a subunit of slightly lower *Mr* (67,000-73,000), designated as x- and y-type, respectively. Bread wheat cultivars contain from three to five HMW subunits when separated by SDS-PAGE; usually, the *Glu-D1* locus encodes two subunits, the *Glu-B1* two or one and the *Glu-A1* locus one or none. When only one subunit is present at the *Glu-B1* and *Glu-A1* loci, this is always an x-type (Payne 1987).

The LMW-GS are coded by genes on the short arms of chromosomes 1A, 1B, and 1D. The loci are designated as *Glu-A3*, *Glu-B3*, and *Glu-D3*. Among a collection of 222 hexaploid wheats from 32 countries, 20 different electrophoretic band patterns (LMW-GS blocks) were detected: six for the *Glu-A3* locus, nine for the *Glu-B3* locus and five for the *Glu-D3* locus (Gupta and Shepherd 1990). Chromosome 1A encodes only a few LMW-GS, and many cultivars do not exhibit any LMW-GS encoded by *Glu-A3*. In contrast, a great deal of polymorphism exists for LMW-GS encoded by chromosome 1B. The number of electrophoretic band combinations found for LMW-GS is much lower than expected based on random association of gene loci products, indicating that genes coding for these bands are closely linked.

The genes coding for most γ- and ω-gliadins have been mapped on the short arms of chromosomes 1A, 1B and 1D at the *Gli-A1*, *Gli-B1* and *Gli-D1* loci, respectively. The genes coding for most α- and β-gliadins occur on the short arms of group 6 chromosomes at the *Gli-A2*, *Gli-B2* and *Gli-D2* loci (Payne 1987). Each gliadin locus was found to control the synthesis of several jointly inherited gliadin components (blocks), and 12-25 alleles have been assigned to each locus (Metakovsky 1991). There is also tight linkage between gliadin genes and genes coding for the LMW glutenin subunits on group 1 chromosomes (Gupta and Shepherd 1993).

Fractionation, Purification of Wheat Flour Proteins and Relationships to Breadmaking Quality

The scientific watershed for the study of wheat proteins derives from the comprehensive fractionation scheme developed by Osborne (1907). Wheat proteins were classified into four major fractions based on their differential solubility by sequential solvent extraction.

i) albumins - soluble in water;

ii) globulins - soluble in salt solutions;

iii) gliadins - soluble in 70% to 90% ethanol;

iv) glutenins - soluble in dilute acid or alkali.

Chen and Bushuk (1970) modified the classical procedure to give five fractions instead of the original four. In the modified Osborne procedure, the glutenin fraction is divided into two subfractions: one that is soluble in 0.05 N acetic acid and one that is insoluble in this solvent. The Osborne-type procedures have been considered to provide a sound basis for the separation of wheat proteins into their main groups. However, wheat flour proteins are heterogenous and have strong tendencies to aggregate. The literature has shown that it is almost impossible to obtain clean fractions by solubility fractionation. This is especially true for gliadin and glutenin proteins, which interact to form gluten.

Glutenin in Gliadin Extracts

Beckwith et al (1966) subjected classical gliadin (70% ethanol soluble) to gel filtration chromatography (GFC), which revealed a high molecular weight fraction as an excluded peak. The intrinsic viscosity of this fraction was between that of glutenin (prepared according to method of Jones et al 1959) and classical gliadin. Also, differences were observed between the amino acid composition of this fraction and that of classical gliadin. They concluded that the high molecular weight fraction isolated from classical gliadin by GFC was actually low molecular weight glutenin. Nielsen et al (1968) provided further evidence that the high molecular weight fraction of classical gliadin contained interchain disulfide bonds. The viscosity of this fraction decreased drastically upon disulfide cleavage; also its *Mr* dropped from about 125,000 to 37,000. The predominant low molecular weight fraction (purified gliadin) showed no change in viscosity upon disulfide cleavage.

Bietz and Wall (1973, 1980) further characterized the high molecular weight fraction of classical gliadin and compared it with ethanol soluble glutenin subunits prepared from pure glutenin (Bietz and Wall 1972). Based on electrophoretic data, amino acid composition and N-terminal sequence results, they concluded that the polypeptides comprising the high molecular weight gliadin were identical to the ethanol-soluble glutenin subunits. By two dimensional electrophoresis, Jackson et al (1983) clarified this ambiguity concerning the identity of high molecular weight gliadin by showing that the constituent subunits of the high molecular weight fraction of classical gliadin were identical to LMW-GS and quite distinct from the bulk of the major gliadin polypeptides.

Shewry et al (1983) isolated a fraction of so-called aggregated gliadin from the excluded GFC peak of aqueous ethanol soluble gliadins in a solvent containing the denaturant 6.0 M guanidine hydrochloride. The GFC peak material only gave a streak with few discrete bands when separated on SDS-PAGE without reduction. Upon reduction, this fraction resolved into two major components with *Mr* of about 44,000 and 36,000. Field et al (1983a) studied the 50% 1-propanol extracts of ground wheat grain by GFC on a column of controlled-pore glass. SDS-PAGE analysis of the fractions demonstrated that the large molecules from the aqueous propanol extracts contained not only LMW-GS but also HMW-GS.

Autran et al (1987) prepared gliadin extracts from durum semolina using 70% ethanol. It was found that a substantial amount of protein in this extract was excluded from GFC column (Sephadex G-150). On this basis, the excluded fraction was called low molecular weight glutenin. The low molecular weight glutenin contributed 14.4% and 27.1% of the extracted protein from a medium and a good pasta-making durum wheat, respectively, based on densitometry of the electrophoretic patterns under reduced conditions.

Huebner and Bietz (1993) separated classical gliadin from defatted flour into ethanol-soluble glutenin (ESG) and gliadins by low pressure size-exclusion chromatography (SEC). The amounts of each fraction were determined gravimetrically after freeze-drying. The ESG consisted of 20-37% of the total ethanol-soluble protein extracted from flour of different cultivars. The authors indicated a need to consider the importance of this glutenin in functionality.

Gliadin in Glutenin Extracts

The aqueous alcohol insoluble flour protein has been widely referred to as glutenin, which is partly soluble in dilute acid or dissociating agents. However, substantial amounts of monomeric proteins remained in the aqueous alcohol insoluble residue, and were subsequently extracted by dilute acid as glutenin.

Orth and Bushuk (1973) prepared acetic acid soluble glutenin from flour milled from cultivar Manitou according to the modified Osborne fractionation procedure (Chen and Bushuk 1970). Electrophoresis of this glutenin under non-reducing conditions gave an electrophoregram with a relatively high concentration of bands of high mobility. Amino acid composition of Osborne-prepared glutenin differed from that of pure glutenin prepared by pH precipitation. They concluded that the Osborne method yields a glutenin fraction that appeared to be highly contaminated with gliadins, albumins, and globulins.

Bietz and Wall (1975) extracted wheat flour sequentially with 0.04 M NaCl, 70% ethanol, 0.1 N acetic acid, 0.01 N acetic acid-0.2 mM $HgCl_2$, and 0.1 N acetic acid-0.1% 2-mercaptoethanol. From 30 to 51% of the protein extracted with acetic acid or $HgCl_2$, after prior extraction with NaCl and ethanol, was found not to be glutenin. Based on results of SDS-PAGE, the authors concluded that these proteins appeared to be gliadins, albumins and globulins which were probably associated with glutenin through non-covalent bonds. These contaminating monomeric proteins might include some glutenin subunits, since $HgCl_2$ was found to be a reducing agent for glutenin (Danno et al 1975). They concluded that the Osborne-type procedure for separation of wheat protein classes was less successful than commonly supposed at the time.

Dupuis et al (1996) quantified gliadin proteins not extracted with ethanol but subsequently extracted by acetic acid as part of the soluble glutenin fraction in modified Osborne fractionation. Two wheat cultivars of diverse breadmaking quality were examined in that study (Katepwa and Glenlea). It was found that the gliadins represent about 30% and 50% of total acetic acid soluble protein, for the extra strong Glenlea flour and strong Katepwa flour, respectively. It was proposed that the insolubility of these gliadins was due to genotype-specific gliadin-glutenin interactions.

Byers et al (1983) found that 50% 1-propanol was much more efficient in extracting wheat flour proteins than 70% ethanol. Based on this information, Marchylo and co-workers (Marchylo et al 1986, 1989; Kruger et al 1988) developed a sequential extraction scheme that separated groups of flour proteins for subsequent analysis of quality differences using RP-HPLC and electrophoresis. Their sequential extraction procedure consisted initially of removing albumins and globulins with 0.5 M NaCl. The residue was treated first with 50% 1-propanol and subsequently with 50% 1-propanol containing dithiothreitol (DTT) to reduce glutenin and extract HMW and LMW subunits. Finally, 50% 1-propanol containing DTT and 1% acetic acid was used to further solubilize any remaining HMW- and LMW-GS. Still the major wheat endosperm protein classes were cross-contaminated. First, although gliadins were preferentially extracted with 50% 1-propanol, polymeric glutenin containing both HMW- and LMW- subunits were also extracted. The HMW-GS can be recovered from the reduced 50% 1-propanol extracts by a selective precipitation procedure with 1-propanol to a concentration of 60% (Marchylo et al 1989), but LMW-GS remaining in the supernatant were contaminated by

gliadins. Secondly, gliadins, especially ω-gliadins, were not completely removed by 50% 1-propanol even after three extractions, and were still present together with glutenin subunits in the subsequent propanol-DTT extracts.

Fu and Sapirstein (1996) proposed that a major contributing factor to the cross-contamination of gliadin and glutenin in Osborne-type fractionation was the use of salt, which would promote the aggregation of gliadin with glutenin.

The fractionation based on solubility of highly complex and heterogenous wheat proteins was further complicated by taking into account the numbers of variables in an extraction procedure: the solvents used; the sample to solvent ratio; the number of extractions, and their timing with any one solvent; and extraction temperature. These variables of extraction conditions can often result in different extractability and different polypeptide composition of the solubilized material (Byers et al 1983). Because different extraction procedures were used by research groups, an accurate comparison of the results of different groups is virtually impossible (Schofield and Booth 1983).

Purification of Glutenin

A great deal of research has been carried out on the physicochemical properties of glutenin in an attempt to elucidate its structure in relation to functionality in breadmaking. It is extremely important to have pure starting material for subsequent molecular and structural characterization. Many different procedures have been suggested in the literature for preparing relatively pure glutenin.

Jones et al (1959) described two methods for the preparation of glutenin. For both

methods, flour was first defatted with water-saturated n-butanol and a gluten ball was prepared by washing the dough in 0.1% NaCl solution. The first method involved dispersing the washed gluten in 70% ethanol to dissolve the gliadins. The suspension was then centrifuged and the residual protein was defined as glutenin. In the second method, often referred to as a pH precipitation procedure, the washed gluten was dispersed in 0.01 M acetic acid and ethanol was added to a final concentration of 70% in the suspension. The pH was then adjusted to 6.5 with 2 N NaOH. The protein that precipitated at 0°C to 4°C and separated by centrifugation was defined as glutenin.

Woychik et al (1964) prepared glutenin by repeated (twice) application of the pH precipitation procedure of Jones et al (1959). They redissolved the glutenin precipitate in 0.01 M acetic acid, and then added ethanol to a concentration of 70% and adjusted the pH to 6.7 with NaOH to reprecipitate the glutenin.

Ewart (1972) used a procedure for preparing glutenin similar to the above-cited first method of Jones et al (1959). The total residue remaining after sequential extraction of gluten with 0.04 M NaCl and 70% ethanol was considered to be glutenin.

Orth and Bushuk (1973) prepared glutenin by pH precipitation followed by further purification using ion exchange chromatography. They extracted flour with a solvent comprising 0.1 M acetic acid, 3 M urea, and 0.01 M cetyltrimethylammonium bromide (AUC) (Meredith and Wren 1966). After centrifugation, the supernatant was adjusted with ethanol to a concentration of 70% and its pH was increased to 6.4 using 1 N NaOH. The resulting precipitate was centrifuged, dispersed in 0.01 M acetic acid, dialysed against distilled water, and finally freeze-dried to give "crude" glutenin. The crude

glutenin was purified by dispersion into AUC solvent with sulfoethyl-sephadex ion-exchanger. The suspension was then centrifuged, the supernatant dialysed against distilled water, and finally freeze-dried to give purified glutenin. The yield of glutenin was very low (10% of total flour protein) due to losses in purification.

Wasik and Bushuk (1974) purified glutenin from AUC extracts of flour by stepwise precipitations with ammonium sulfate. The proteins obtained from the first precipitation with ammonium sulfate were redissolved in AUC solvent and precipitated once more with ammonium sulfate to give purified glutenin.

Bietz and Wall (1975) used a series of solvents (acetic acid followed by acetic acid-mercuric chloride, and finally with acetic acid-β-mercaptoethanol (β-ME)), to solubilize total glutenin (residue proteins after salt and alcohol extractions). Fractions from the acetic acid and acetic acid-mercuric chloride extracts were further purified by the pH precipitation procedure of Jones et al (1959). Bietz and Wall (1975) concluded that carefully defined conditions, including a precipitation step, must be used to produce a suitable purified preparation of glutenin for any study.

Graveland et al (1982, 1985) developed a combined method to prepare various fractions of glutenin using extraction, ultracentrifugation, precipitation and gel filtration. Flour was suspended in 1.5% SDS solution and ultracentrifuged. Three distinct layers were formed at the bottom of the centrifuge tube. They defined the top layer as SDS-insoluble "glutenin I" (gel protein). After adding ethanol to the SDS soluble fraction to a concentration of 70%, a precipitate was formed which was isolated by centrifugation. After removing the globulins and pentosans from the precipitate by distilled water or

0.01 M NaCl, a "glutenin II" fraction was extracted with 0.05 M acetic acid. The acetic acid-soluble glutenin II fraction was separated into two subfractions, called "a" and "b", by gel filtration on a Sepharose CL-4B column using 1.5% SDS solution as eluent. The SDS- and ethanol-soluble "glutenin III" fraction was isolated from the ethanol-SDS supernatant by cooling the supernatant to -30°C. The precipitate obtained after centrifugation consisted of glutenin III and gliadins. The latter fractions were separated by gel filtration on a Sephadex G-100 column with 1.5% SDS solution as eluent. The first (void), second, and third peaks were identified as glutenin IIIb, glutenin IIIa, and gliadins, respectively.

Khan and Bushuk (1979) used SDS-PAGE under non-reducing conditions to examine the purity of glutenin preparations. Their criterion of purity was that unreduced glutenin should not migrate into the resolving gel as discrete bands during SDS-PAGE. They examined two crude glutenin preparations (modified Osborne acetic acid-soluble, and 2-chloroethanol extracts of modified Osborne residue protein) and four purified glutenin preparations isolated according to four published methods (Jones et al 1959, Orth and Bushuk 1973, Bietz and Wall 1975, Bietz et al 1975). Their SDS-PAGE results showed that all the unreduced glutenin preparations contained many prominent protein components of *Mr* 68,000 and lower. Accordingly, all the above methods resulted in contaminations of monomeric protein with glutenin presumably through non-covalent interactions. It is noteworthy that all the above procedures incorporated salt as one of the first extraction steps.

More recently, Sapirstein and Suchy (1996) examined the protein composition of

SDS-insoluble glutenin (i.e. gel protein). It was found that this fraction of glutenin contained substantial amounts of gliadins, whose proportion varied inversely with loaf volume potential of wheat flour samples.

Recently, research on wheat glutenin was stimulated by the interest in the possible relationship between LMW-GS composition and breadmaking quality. To analyze LMW subunit composition, it is necessary to first remove monomeric proteins which co-migrate or co-elute with LMW-GS during SDS-PAGE or RP-HPLC.

Burnouf and Bietz (1989) extracted wheat flours with various solvents to develop a rapid procedure to isolate glutenins for RP-HPLC. They investigated 11 solvents to purify glutenin by removing monomeric proteins from defatted and nondefatted flours. Dimethyl sulfoxide (DMSO) was found to be an excellent solvent for all monomeric proteins. Their DMSO extraction procedure (two cycles of extraction) first quantitatively removed the monomeric proteins from flour. The residue was then washed with 70% ethanol to remove the residual DMSO. Glutenin remaining in the residue was extracted with 8 M urea containing 5% β-ME as a reducing reagent and alkylated prior to RP-HPLC analysis.

Gupta and MacRitchie (1991) used the procedure of Burnouf and Bietz (1989) to remove all the monomeric proteins from flour. They found that the residue contained some polymeric albumins and globulins in addition to glutenin. Glutenin subunits free of albumin and globulin proteins were isolated by dispersing the residue in 70% ethanol containing 2% β-ME at 65°C. The hot ethanol selectively dissolved the glutenin subunits but not the albumins and globulins.

Singh et al (1991) reported what may be the most straightforward procedure for glutenin preparation with little or no contamination from other classes of wheat proteins. The procedure was based on the sequential extraction method described by Marchylo et al (1989). They eliminated the salt extraction step in the original method, which created difficulties with the subsequent resuspension of residue due to formation of a cohesive gluten mass during salt extraction. Flour was extracted at least twice with 50% 1-propanol. Subsequently, highly pure glutenin subunits were extracted from the residue with 50% 1-propanol containing 1% DTT.

While the procedures of Gupta and MacRitchie (1991), and Singh et al (1991) appear to be successful in obtaining relatively pure glutenin in essentially one step, they also result in the extraction of some glutenin. The nature and amount of this soluble glutenin has largely been ignored in the literature, with the exception of Bietz and Wall (1973, 1980) who studied the qualitative nature of glutenin soluble in ethanol.

Size-based Separation of Flour Proteins

Because of the complex heterogeneity of wheat proteins, it is difficult to effect a sharp separation using differential solubility. As a consequence, a popular approach has been to separate wheat proteins based on molecular size differences. Studies on the molecular weight distribution of glutenin and the relative proportion of glutenin in total flour proteins have been important parts of the overall attempt to elucidate the relationship between protein structure and breadmaking quality. Research in this area has been closely related to the progress in solubilizing wheat storage proteins, and also

improvements in established and new techniques for the study of protein molecular weight distributions.

Meredith and Wren (1966) introduced the well-known AUC solvent for solubilization and chromatography of wheat flour proteins. They reported that this solvent extracted about 95% of the total protein of a commercial flour. They then separated the flour protein in the AUC extract by GFC on Sephadex G-200 medium, which produced four peaks, representing glutenins, gliadins, albumins and non-protein constituents, respectively.

Huebner and Wall (1976) extracted proteins with AUC solvent from several flours with diverse dough strength. The proteins were subjected to GFC on Sepharose 4B, and glutenins were separated into two fractions termed I (high molecular weight) and II (low molecular weight). The ratio of glutenin I to glutenin II was generally higher for good breadmaking cultivars. These findings are in agreement with the earlier work of Orth and Bushuk (1972) who showed that the acetic acid soluble and insoluble glutenin were negatively and positively correlated, respectively, with loaf volume.

Payne and Corfield (1979) obtained a much lower flour protein extraction (75%) than that claimed by Meredith and Wren (1966) for the same AUC solvent. They fractionated the AUC extracts of flour using GFC in cross-linked Sepharose. The glutenin eluted as a very broad fraction, indicating that it had a wide molecular weight distribution (200,000 to several million) in AUC solvent. The protein elution profile was similar to that obtained by Huebner and Wall (1976).

Danno et al (1974) compared 12 solvents for the solubilization of wheat proteins

at the same extraction conditions. The 1% SDS solution (pH 6.8) gave the highest extraction (75%), slightly better than the AUC (72%) under the conditions used. Later, Danno (1981) reported another approach to solubilizing flour proteins by twice extracting flour with 0.5% SDS (pH 7.0). About 77% of the total flour nitrogen was solubilized by the first extraction. The remaining proteins in the residue, mainly glutenin, were almost completely extracted by the second extraction by stirring with a blender.

Bottomley et al (1982) showed that 2% SDS-0.1 M Tris/HCl buffer (pH 8.0) extracted wheat flour proteins (95%) more efficiently than any other solvent including AUC. The use of this solvent also gave better resolution of the extracted proteins during GFC (Sepharose CL-4B). It was found that the ratio of the areas under the excluded peak (high molecular weight glutenin) and the following flat peak (low molecular weight glutenin) significantly correlated with breadmaking quality of the flours. This result was in agreement with that of Huebner and Wall (1976), i.e. part of the quality factor of a flour is determined by the molecular size distribution of the glutenin protein. They suggested that SDS extraction/gel filtration in conjunction with SDS-PAGE was a powerful tool for the detailed analysis of flour protein.

Field et al (1983b) developed a modified AUC solvent, i.e. lower acetic acid concentration and higher concentrations of urea and detergent compared to the original AUC solvent of Meredith and Wren (1966). The modified AUC solvent was then used for solubilization and fractionation of gluten extracts. Two peaks were resolved by SEC of the extracted gluten proteins on a column of controlled pore glass. The first peak, eluted at the void volume and contained polymeric proteins (mainly glutenin) with a wide

range of molecular weights. The second peak contained monomeric proteins (mainly gliadins). The relative amount of protein (polymer/monomer) present in the two peaks was found to be correlated to the breadmaking quality.

Gao and Bushuk (1992, 1993) reported that essentially all of the glutenin prepared from wet gluten could be solubilized with a solvent comprising 6 M urea and 6% SDS by prolonged incubation (24 h) at an elevated temperature (50°C). This solvent was then used to extract gluten proteins from *Glu-1* null wheat lines whose HMW-GS composition ranged in number from five to no subunits. The GFC elution profile (monitored at 280 nm) for the genotype that contained no HMW-GS was essentially the same as that of the control that contained all five HMW-GS, indicating that polymers formed from only LMW-GS were of the same average size as those comprising both HMW- and LMW-GS. The implication of this result is that the contribution of glutenin to intercultivar differences in breadmaking quality depends on properties other than the differences in molecular weight distribution.

GFC has been one of the most useful techniques for the separation and comparison of wheat flour proteins. In spite of its wide use, the conventional technique is subjected to some significant disadvantages. First of all, it is slow, most separations can take one or more days to complete. Column beds are not physically strong, necessitating very low flow rates to avoid compression. It is often difficult to reproduce gel filtration results, and peak resolution is generally poor.

In the past decade, the evolution of SE-HPLC for analysis of wheat flour proteins offers clear advantages (Bietz 1986). The chromatographic columns, based largely on

silica media, have small particle sizes and are physically and chemically stable, resulting in rapid and relatively high-resolution separations. Very small samples can be analyzed, and since flow rates are very constant, reproducible results are generally obtained that can be easily and accurately quantified. The major advantages of SE-HPLC are speed (most separations take only 20-30 min), and the automated nature of most systems which can operate under computer control.

Accordingly, SE-HPLC has been widely used to relate the quantity of polymeric and monomeric wheat protein fractions and the size distribution of polymeric proteins to breadmaking characteristics of wheat flour. Bietz (1984) analyzed unreduced SDS extracts from 19 flour samples of diverse dough strength by SE-HPLC. He found that the magnitude of the excluded peak (glutenin) was inversely related to flour strength. Significantly more high molecular weight protein, characteristic of glutenin, was present among SDS-soluble proteins of weak flours than those of strong flours. He explained this on the basis that strong wheats have less acetic acid or SDS extractable glutenin which in line with previous results (Orth and Bushuk 1972; Moonen et al 1983). In another set of wheats, Huebner and Bietz (1985) found the relationship between the amount of high molecular weight glutenin and dough mixing strength to be direct, similar to the gel filtration results of Huebner and Wall (1976) and Bottomley et al (1982).

Lund and MacRitchie (1989) used SE-HPLC to characterise 10 gluten protein fractions (prepared by a fractional extraction procedure using dilute HCl solutions of progressively decreasing pH) from each of two wheat cultivars differing in dough strength. The proportion of glutenin in each fraction was estimated by summing the

proportion of each fraction that was insoluble in 2% SDS solution and the soluble portion of *Mr* greater than 80,000 deduced from the chromatograms. They derived two general conclusions from their results. Firstly, with increasing fraction number (decreasing pH), the proportion of glutenin rose and this paralleled the effects of the fractions in increasing dough development time and loaf volume. Secondly, and consistent with the first result, a comparison between two varieties showed that the stronger variety had a greater proportion of glutenin than its poor performing counterpart.

Dachkevitch and Autran (1989) using SE-HPLC found an inverse relationship between the amount of HMW glutenin (excluded peak) and flour quality. They chromatographed 2% SDS extracts of unreduced proteins from flours milled from grain samples grown in three different years. The analysis was mainly based on the molecular weight distribution of "large aggregate" (excluded fraction F1) and "intermediate aggregates" (F2). A much lower percentage of the F1 fraction was observed in samples of higher baking strength than those of lower baking strength. This resulted in a significant negative correlation between the amount of fraction F1 and baking strength. In their experimental conditions, the 2% SDS extraction rates ranged between as low as 55% to 90% on a total protein basis. The polymeric proteins were distributed among three fractions, F1, F2 and the insoluble residue.

Singh et al (1990a) showed that sonication of a flour suspension in 2% SDS can provide close to complete extraction of proteins. Total unreduced proteins from 15 flours of diverse breadmaking quality were extracted in this way, and fractionated by SE-HPLC (Singh et al 1990b). Chromatograms yielded three peaks, which corresponded in order

of elution to glutenin (excluded peak), gliadins, and albumins/globulins, respectively. They found that the relative quantity of glutenin was highly positively correlated with loaf volume, extensigraph dough resistance and extensibility, and mixograph peak development time of the flours (r = 0.72, 0.84, 0.84, and 0.84, respectively; $p <$ 0.003-0.0001). The SE-HPLC procedure used by these authors, however, gave considerable overlap among different size class proteins. This procedure was improved by using 0.5% SDS in 0.05 M phosphate buffer, pH 6.9, to extract the protein from flour, and 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid as the elution solvent (Batey et al 1991).

Gupta et al (1992) applied the modified SE-HPLC procedure of Batey et al (1991) to analyze the protein composition of 15 wheat cultivars grown at six different nitrogen levels. They showed that as flour protein increased for a given cultivar, the proportion of glutenin (peak 1) remained constant, the proportion of gliadin (peak 2) increased, and the proportion of albumin/globulin (peak 3) decreased. Only the proportions of glutenin showed consistent relationships with different quality parameters.

Gupta et al (1993) examined the protein composition of three genetically diverse sets of wheat lines by SE-HPLC. Polymeric protein as a percentage of total flour protein did not show significant relationships with dough properties (as measured by extensigraph and mixograph) in some of the wheat sample sets. They measured the size distribution of polymeric protein (mainly glutenin) indirectly. The total polymeric protein, based on its extractability in 0.5% SDS without sonication, was separated into two size groups (extractable and unextractable). The unextractable polymeric protein was then solubilized

by sonication and quantified by SE-HPLC and the percentage of unextractable polymeric protein in total polymeric protein or in total protein was used as a measure of protein size distribution. They found that the portion of the total polymeric protein unextractable in 0.5% SDS solution was very strongly positively correlated with dough strength properties, and accounted for the variation in these parameters to a much greater degree than did the percent total polymeric protein in protein or in flour. This finding is generally consistent with the earlier findings (Orth and Bushuk 1972) because sonication brought the otherwise insoluble glutenin into solution.

Since the work of Gupta et al (1993), the SE-HPLC - sonication - SDS extraction procedure has been frequently used to measure the relative size distribution of the polymeric or total protein in flour (Cornec et al 1994; Gupta and MacRitchie 1994; Popineau et al 1994; Gupta et al 1995; Ciaffi et al 1996). A significant drawback of this procedure is that sonication can substantially reduce the size of native glutenin. Surprisingly, about half of the sonicated glutenin analyzed by SE-HPLC had *Mr* below 158 kDa (Gupta et al 1993), the size of which equals an oligomer of e.g. only two HMW subunits or four LMW subunits. The results so obtained are no longer representative of native glutenin which are thought to have *Mr* ranging from a few hundred thousand to many millions (Wahlund et al 1996).

Conclusions about flour protein composition and breadmaking quality reached in various studies based on SE-HPLC are not in agreement. The origin of the discrepancies may be partly explained by the conditions of protein extraction and by extraction rate; a smaller proportion of glutenin is solubilized from strong flours than from weaker ones.

In none of the studies cited above was complete separation of polymeric and monomeric flour proteins achieved, although various media and SE-HPLC procedures were tried. In addition to the difficulty in solubilizing native glutenin, GFC and SE-HPLC lose their resolution above a certain molecular size, resulting in poor or no fractionation of glutenins of different molecular weights. Two recent papers (Stevenson and Preston 1996, Wahlund et al 1996) demonstrated the potential of field-flow fractionation to size-fractionate the ultra-high molecular weight glutenin proteins, and to reach beyond the size range which GFC and SE-HPLC have their exclusion limits. Even in these studies, sonication was used to achieve a complete solubilization of large sized glutenin. Accurate determination of the size distribution of native glutenin is an analytical challenge that remains to be resolved. The multistacking gel procedure in SDS-PAGE developed by Khan and Huckle (1992) appears to be useful for characterizing native (nonreduced) soluble glutenin with respect to the size differences of its molecules.

Fractionation and Reconstitution/Fortification Studies to Assess the Functionality of Gluten Proteins

Several approaches are available for tackling the question of what constitutes the basis of baking quality in wheat flours. One of the most direct ways to bridge the gap between functionality and composition is by fractionation and reconstitution/fortification techniques. This approach involves separation and fractionation of flour components and their fractions. Each component (or fraction) is then evaluated by varying its amount in a given flour or by interchange between flours of different baking quality to establish the

role of each and to find which are the fractions responsible for differences in quality. An important consideration of this approach is that neither the isolation nor the reconstitution procedures impair the functional properties of the components.

Fractionation and Reconstitution/Fortification of Gluten Proteins

In one of the earliest studies of this sort, Aitken and Geddes (1938) prepared dry glutens by employing a drying temperature of 32°C and a rapid air flow. After reducing the glutens from weak, intermediate, and strong wheats to a flourlike fineness, they fortified the original flours with corresponding glutens in such a manner as to equalize their protein contents. Thus they expected to compare more accurately their relative gluten qualities. The additions of dry gluten resulted in significant improvement in dough-handling properties and loaf volume. While increasing the protein content of weak flours by the addition of their own dried gluten resulted in marked general improvement, the addition of gluten from a strong wheat produced more pronounced strengthening effects. Later, Aitken and Geddes (1939) prepared seven flours ranging in protein content from 10.5% to 22.7% by enriching the lowest-protein flour with dried gluten. These protein-fortified flours were employed to study the relationship between loaf volume and protein content over a wide range without introducing differences in protein quality. Significant increases in dough strength and loaf volume associated with increasing levels of gluten protein demonstrated the importance of protein quantity effects.

The first systematic fractionation and reconstitution study was carried out by Finney (1943). He fractionated three flours, representing a wide range in quality

characteristics, into starch, gluten, and water-soluble components. Components from one cultivar when recombined in the original proportions, resulted in the loaf volume equal to that made with the original, unfractionated flour. Interchange of components between flours of different baking quality showed that gluten protein was the component almost wholly responsible for inherent differences.

Once the key role of gluten proteins in governing varietal differences in breadmaking potential was established, the next logical step was to identify the gluten component(s) responsible for those differences. The separation and reconstitution of starch, gluten protein, solubles and lipid presented no major problems. The greatest challenge occurs when attempts are made to fractionate and reconstitute gluten proteins. Many workers have used different fractionation/fortification procedures to identify gluten component(s) responsible for intercultivar differences in mixing properties and baking potential of wheat flours.

Hoseney et al (1969a, 1969b) showed that separation by ultracentrifugation of the two main fractions of gluten, into a sediment containing glutenin and a supernatant rich in gliadin did not impair the breadmaking properties of gluten components after reconstitution. They solubilized gluten in 0.005 M lactic acid (pH 4.7). Some 5-8% of the total gluten protein remained insoluble, and when this insoluble portion was interchanged between a good and poor variety flour, loaf volume potentials were unchanged. Gluten soluble at pH 4.7 was fractionated by ultracentrifugation at 1000,000*g* for 5 h. Approximately 15% of the protein was recovered as sediment, 85% remained in the supernatant. When the sediment and supernatant were interchanged between a good

and poor baking flour, the differences in loaf volume was accounted for by the supernatant. The authors suggested that if it is assumed that the glutenin contained in the supernatant fraction behaves similarly to the glutenin of the insoluble protein and the sediment fraction of the soluble (i.e. it does not account for loaf volume differences between flours), then differences in loaf volume potential would reside in the gliadin fraction.

Hoseney et al (1969b) fractionated glutens into gliadin and glutenin with 70% ethanol. The ratio of gliadin to glutenin was essentially constant at 53% gliadin and 47% glutenin for four wheat cultivars that varied widely in breadmaking quality. Reconstitution of glutenin fractions of good- and poor- quality cultivars with a fixed gliadin-rich fraction showed that the gliadin protein controls the loaf volume potential of a wheat flour. Similar reconstitution studies showed that the glutenin fraction governed the mixing requirements of a wheat flour.

Gliadin and glutenin behave quite differently in regard to their contributions to dough properties; a behaviour that can serve as a basis for classification. Lee and MacRitchie (1971) fractionally extracted gluten protein from flour using urea solutions of increasing concentration and assessed the functional properties of the fractions by addition to a base flour and evaluation of mixograms and alveograms. The early-extracted fractions caused weakening of the dough. Beyond a certain point in the successive extraction procedure, extracted fractions produced the opposite effects. The gel filtration profile of the fractions showed that the proportion of high molecular weight protein increased with each successive extraction. MacRitchie (1973) produced flours with

predictable properties of strength and elasticity by varying the proportions of early and later extracted gluten protein in a given flour.

MacRitchie (1978) separated glutens from two flours of different baking performance each into two fractions by extraction with 0.1 M acetic acid. The soluble and insoluble fractions contained approximately 60% and 40% of the total gluten protein, respectively. Interchange experiments indicated that the quality (baking potential) factor resided in the insoluble gluten fraction (glutenin). In subsequent work, MacRitchie (1980) used an improved protein fractionation by applying dilute hydrochloric acid as extractant and neutralizing the acid with sodium hydroxide immediately after separation of fractions. It was found that the same concentration of acid extracted different amounts of protein from glutens of two flours chosen on the basis of their differing baking performance. To overcome this problem, calibrations of acid concentration as a function of quantity of protein extracted were made for both gluten samples. Acid concentrations were then chosen so as to extract equal proportions of protein from the two gluten samples. When 33% of the total gluten protein from each flour was extracted into the supernatant and these fractions interchanged between the flours, no effect on the original loaf volume was observed. Interchange of the sediment fractions (67% of total gluten protein) accounted for all the differences in loaf volume. However, when the acid concentration was increased to extract 70% of the gluten protein into the supernatant, interchange of fractions showed that the supernatant fraction accounted for 65% of the loaf volume differences and the sediment (30% of total gluten protein) accounted for about 35%. The additional protein extracted in the supernatant (about 40% of the total

gluten protein) accounted for 65% of the loaf volume difference, assuming the contributions from fractions are additive. MacRitchie (1980) concluded that the earliest extracted portion of the gluten protein (mainly gliadins) is not very important in explaining baking quality differences, and the gluten quality is concentrated in the protein of intermediate solubility (soluble glutenin).

Another study of the effects of acid-soluble and acid-insoluble gluten proteins on rheological and baking properties of wheat flours was carried out by Preston and Tipples (1980). The 0.05 M acetic acid soluble and insoluble gluten protein fractions of a hard red spring wheat flour made up approximately 85% and 15% of the total gluten protein, respectively. The two fractions were tested by addition of varying amounts to two base flours. Mixograms and farinograms of flours to which fractions were added suggested that dough-strengthening effects were mainly due to proteins in the acid-soluble fraction, whereas the acid-insoluble protein at higher levels had a slight dough weakening effect. Addition of increasing amounts of acid-soluble gluten progressively increased loaf volumes of both flours. In contrast, increasing additions of the acid-insoluble protein caused progressive lowering of loaf volume.

MacRitchie (1985) conducted a systematic study of the different steps in the separation, fractionation, and reconstitution of wheat flours to determine where detrimental changes to functional properties might arise. Guidelines for preserving protein functionality during fractionation were described. Some of the important precautions that need to be observed are the extraction of non-starch flour lipid with an appropriate solvent (e.g. chloroform) as a first step, the washing out of gluten at an

optimum temperature (15°C), minimisation of contact time for gluten proteins with acid solution (2 min homogenisation in dilute HCl), neutralisation of supernatant and sediment to constant pH (e.g., 5.8), and grinding of freeze-dried fractions to a suitable particle size (< 250 μm). Numerical results were given for two flours as a guide for separating, fractionating and reconstituting flour components with complete recovery of original flour functional properties. Separation of the gluten protein of each flour into two approximately equal fractions followed by interchange of fractions between the reconstituted flours, showed that quality differences reside mainly in the more acid-insoluble fraction (glutenin) of the gluten protein.

MacRitchie (1987) fractionated proteins from glutens of six wheat varieties (three high, three low baking performance), into either nine or ten fractions by successive extraction with dilute HCl of progressively decreasing pH. Fractions were added to base flours to increase protein levels by 1%. The fortified flours were assessed in terms of their mixograph peak development times and loaf volumes. Early extracted fractions (fractions 1-4) which had high proportions of gliadin, decreased mixing requirements and slightly depressed loaf volume. The later extracted fractions (fractions 5-8) had a high concentration of glutenin and produced large increases in dough development times and also loaf volume. This trend was reversed by the latest-extracted fractions (fractions 9-10), including the final residue. Results of amino acid analyses suggested that the latest fractions contained nongluten polypeptides. Using 0.75 M NaCl, more protein (believed to be the main protein present in the latest fractions) was extractable from glutens of the poorer flours than those of the better-performing flours. MacRitchie (1987) concluded

that the relative proportion of these globulin-type proteins to the glutenins appears to be important in determining baking quality.

Chakraborty and Khan (1988a, 1988b) investigated procedures that have been used in three different laboratories (Hoseney et al 1969a, 1969b; Chen and Bushuk 1970; MacRitchie 1978) for preparing flour fractions for use in reconstitution studies. Their conclusions involved the following: 1) the use of 70% ethanol as extractant, although giving a relatively sharp separation of gliadin, was unsatisfactory for reconstitution studies, because the functional properties of the protein were altered, 2) the heterogeneity of the fractions produced made it invalid to use the terms gliadin and glutenin; the fractions were shown by SDS-PAGE to be mixtures of these two main classes of protein, 3) the origin of differences in loaf volume potential resided in the glutenin component of the flours; exchanging the gliadin and glutenin fractions between two cultivars of poor and good breadmaking quality showed that the fractions that contained larger amounts of glutenin proteins gave the highest positive responses to loaf volume.

Weegels et al (1994a) developed a pilot scale protocol for cation exchange fractionation of 70% ethanol soluble gliadins. Gliadins were separated into five fractions differing widely in composition. Isolated fractions were evaluated for their effects on breadmaking quality using a pan loaf baking test. Additions of all individual fractions to a base flour improved loaf volume, but to a different extent. They compared the fortification results with those of a statistical study performed earlier (van Lonkhuijsen et al 1992). A good agreement was observed between the actual increases in loaf volume caused by addition of flour of the five fractions and the increase predicted by a statistical

equation using only the quantity of some gliadins to predict loaf volume. They found that hydrophobic gliadin fractions were more effective in increasing loaf volume than hydrophillic fractions.

Gupta et al (1994) isolated protein fractions rich in HMW-GS (*Glu-1*) polymers, LMW-GS (*Glu-3*) polymers, gliadins (*Gli-1*, *Gli-2*) and secalins (*Sec-1*) from genetic lines lacking specific groups of wheat proteins and commercial wheat flours. Each fraction was extracted at a particular pH from the flour suspension using dilute HCl, neutralized using NaOH, and freeze-dried (MacRitchie 1987). The fractions were added, at a constant protein level, to a base flour and their effects on dough properties were measured using a 2-g mixograph and 50 g extensigraph. The results suggested that polymers of *Glu-1* subunits had significantly greater positive effects on dough development time and maximum dough resistance than the polymers of *Glu-3* subunits. On the other hand, gliadin and *Sec-1* fractions showed negative effects on dough strength.

Fido et al (1994) prepared a total gliadin fraction from Chinese Spring wheat by both ethanol and dilute HCl (pH 5.3) extraction. Individual groups of gliadins (α-, β-, γ-, ω_1-, and ω_2) were purified using ion-exchange and SEC and identified by lactate-PAGE. Their effects on the mixing properties of flours were determined on a 2-g mixograph by adding increasing amounts of each fraction to two base flours. The addition of individual gliadin polypeptides resulted in weakening effects on mixing properties of both base flours. Differences in the size of these effects were observed and the order of the weakening effects were fractions specified as ω_1- > ω_2- = α- = β- > γ- gliadins.

Chemical Incorporation of Gluten Polypeptides into Polymers

Glutenins are comprised of two sets of individual polypeptides (HMW and LMW subunits), linked together by disulfide bonds into very large molecules. Understanding of the functionality of glutenin subunits is mainly based on correlation studies. These are valid for identifying marker proteins but do not necessarily indicate cause-and-effect relationships. Recently, Bekes et al (1994a) have devised a novel procedure for testing more directly the contribution of individual glutenin subunits in doughs mixed in the 2-g mixograph. The procedure involves the partial disruption and reformation of disulfide bonds of flour proteins in dough to ensure incorporation of the added polypeptide. By carefully selection of the type and concentration of oxidising agent, the mixing properties of partially reduced doughs can be fully restored by reoxidation.

By SDS-PAGE and SE-HPLC, Bekes et al (1994b) showed that HMW subunit *Glu-1Bx* 20 (isolated from wheat) was incorporated into the glutenin polymer after adding 7-10 mg to 2 g of flour treated according to the reduction/reoxidation procedure. In contrast, when the subunit was added to dough without the reduction/reoxidation treatment, ten times less of the HMW-GS was incorporated. After subunit incorporation, mixing time and dough resistance increased. Similar results (Bekes and Gras 1994) were obtained with HMW-GS 2, 5, 10, 12 expressed in *E. coli*. X-type subunits 2 or 5 were more effective in increasing the mixing time than y-type subunits 10 or 12. Mixing studies with incorporated 5+10 and 2+12 pairs showed that 5+10 had a larger strengthening effect than the same amount of 2+12. Changing the ratio of pairs incorporated, it was found that at the 1:1 ratio, maximum effects on mixing properties

could be obtained. The effects of incorporation of subunits 1Dx with 1Dy were much stronger than those of either 1Dx with 1Dx or 1Dy with 1Dy, indicating that interaction between the subunits occurred in polymer formation.

Bekes et al (1995) found that differences in the dough strengthening effect for incorporation of combined mixtures of HMW-GS 5+10 and 2+12 were relatively small compared to the differences that were observed between individual x- and y-type subunits. No comments were offered to explain this result. They concluded that the size of a subunit appears to be the important property that determines its effect on mixing properties; the larger the subunit that is incorporated, the larger the mixing time to peak development.

Szabo et al (1995) examined the reoxidation behaviour of purified glutenin subunits by comparing the kinetics *in vitro* of the polymerisation and by measuring the size distribution of the resulting polymers. HMW-GS 7 and 8 were derived from wheat flour and subunits 2, 5, 10 and 12 were produced by heterologous expression in *E. coli*. Oxidation reactions were carried out in buffered solutions (pH = 8 and 2), using $KBrO_3$, KIO_3, $KMnO_4$, H_2O_2 or gaseous O_2 as oxidising agents. Reaction mixtures were sampled from 0 h to 24 h reaction time. Multi-stacking gel electrophoresis (Khan and Huckle 1992) was used to quantify the remaining monomeric subunits as well as five oligomeric groups with different molecular weight. The relative oxidation rates in forming oligomers were LMW > *Glu-1Dx 5* > *Glu-1Dx 2* > *Glu-1Bx 7* > *Glu-1Dy 10* > *Glu-1Dy 12* > *Glu-1By 8*. Oxidation of x-type subunits was faster than the y-type subunits for each locus. Oxidation of *Glu-1D* subunits was faster than that involving subunits of the *Glu-1B*

locus. Both *Glu-1Dx 5* and *Glu-1Dy 10* oxidised faster than *Glu-1Dx 2* or *Glu-1Dy 12*, respectively. The oxidation of mixtures of x- and y-type subunits was significantly faster than homo-polymerisation of either x- or y-type subunits, resulting in a molecular weight distribution richer in larger polymers, compared to the results of homo-polymerisation of either x- or y-type subunits.

Schropp et al (1995) studied the reoxidation behaviour of HMW-GS with regard to subunit composition, protein concentration, solvent composition and pH, and oxidation time. The reoxidised products were characterized by the determination of the thiol content, the molecular weight distribution, and the subunit proportions. The results demonstrated that HMW-GS could be reoxidised to polymers with molecular weights up to several million. However, the different combinations of subunits, representative for good (7+9, 5+10) and poor (6+8, 2+12) quality, did not influence the kinetics of oxidation and polymerization. Schropp and Wieser (1996) evaluated the reoxidised HMW-GS of different composition by adding to two base flours. The corresponding glutens were characterized by microscale extension tests and compared to glutens from the base flours without addition of any reoxidised subunits. The maximum resistance of gluten was increased and extensibility was decreased by reoxidised HMW subunits. The effect of reoxidised HMW subunits on gluten rheological properties did not seem to depend on the HMW subunit composition.

In summary, conclusions arrived by various reconstitution studies are not in agreement. Some showed that the acid-insoluble glutenin fraction is responsible for the quality differences between cultivars (MacRitchie 1978, 1985; Booth and Melvin 1979);

others that the acid-soluble gluten governed dough strength (Preston and Tipples 1980); still others that gliadin control loaf volume potential of wheat flour (Hoseney et al 1969a, 1969b). It is important to realize that the amounts and composition of protein fractions prepared from a flour or gluten vary depending on the nature and concentration of the extracting solvent and the particular flour or gluten used. Changes in functionality can occur due to denaturation, aggregation and changes in conformation during different steps of fractionation. Proteolysis can also occur during prolonged extraction or dialysis. High-shear extraction procedures (e.g. homogenisation) may damage the structure of the glutenin polymer. Sample treatment is therefore a critical step in fractionation and reconstitution/fortification studies. More work needs to be done in this area in order to provide accurate fundamental information on the relationships between the protein composition and functional properties.

Chapter 3

PROCEDURE FOR ISOLATING MONOMERIC PROTEINS AND POLYMERIC GLUTENIN OF WHEAT FLOUR[1]

Abstract

A new method for fractionation of monomeric (albumins, globulins and gliadins) and polymeric (native unreduced) glutenin proteins of wheat flour has been developed. Proteins were first separated into 50% (v/v) 1-propanol soluble (50PS) and insoluble (50PI) fractions. The 50PI protein was essentially free of monomeric proteins and comprised mainly glutenin; 50PS protein was a mixture of monomeric proteins and polymeric glutenin. Polymeric glutenin in 50PS protein was isolated under non-reducing conditions by precipitation with 1-propanol to a concentration of 70%. Polyacrylamide gel electrophoresis at pH 3.1 (A-PAGE) showed that the precipitated glutenin fraction (70PI) contained some monomeric proteins, mainly ω-gliadins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that no polymeric glutenin remained soluble in aqueous 70% 1-propanol. The fractionation procedure was therefore highly selective. This fractionation procedure, in conjunction with reversed-phase high performance liquid chromatography (RP-HPLC) was then used to examine the flour proteins of two Canadian wheat cultivars (Glenlea and Katepwa) of diverse dough strength. While the amounts of total polymeric glutenin ($\sim$ 50% of flour protein), and the proportions of ω-gliadins in 70PI glutenin ($\sim$30%) were comparable for both cultivars, flour of the very strong mixing Glenlea contained 21% more 50PI glutenin and 30% less 70PI glutenin as determined by Kjeldahl analysis. The ratios of 50PI to 70PI

glutenin were directly proportional to the mixograph dough development times. Results showed that 50PI and 70PI glutenins had the same subunit composition and similar high molecular weight (HMW) to low molecular weight (LMW) glutenin subunit (GS) ratios. The difference in solubility of the polymeric glutenin in 1-propanol is probably due to a difference in molecular size. The results obtained in this study confirmed the importance of both the soluble and insoluble polymeric glutenin in determining flour strength. The protein isolation procedure should be useful for physicochemical characterization of soluble and insoluble glutenin fractions, and for isolating pure glutenin from gliadin-glutenin mixtures.

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Introduction

Recent research on the biochemical basis of breadmaking quality of wheat flour has intensified the need for an accurate and reliable method for separating the polymeric (unreduced or native) glutenin from the monomeric or single polypeptide chain wheat flour proteins (albumins, globulins and gliadins). The rationale for such a separation is twofold. First, the relative amount of polymeric protein in a flour appears to be strongly related to the functionality of the flour in breadmaking (Mecham et al 1962, Tsen 1967, Orth and Bushuk 1972, Tanaka and Bushuk 1973, Huebner and Wall 1976, Field et al 1983b, MacRitchie 1987, Gupta et al 1993). Secondly, after reduction of polymeric glutenin, its subunit composition can be used to predict the breadmaking potential of a wheat cultivar (Payne et al 1979, 1981; Ng and Bushuk 1988; Gupta et al 1989, 1991a).

Many fractionation procedures have been reported to separate the glutenins from the other classes of wheat proteins. Physicochemical approaches have almost invariably been based on the distinction of the very large molecular size of polymeric glutenin. Methods have included ultracentrifugation (Hoseney et al 1969a, Goforth and Finney 1976), gel filtration (Meredith and Wren 1966, Huebner and Wall 1976, Payne and Corfield 1979, Bottomley et al 1982), and size exclusion high performance liquid chromatography (SE-HPLC) (Lundh and MacRitchie 1989, Dachkevitch and Autran, 1989, Singh et al 1990a, Batey et al 1991, Gupta et al 1993). More widely used fractionation methods, because of their apparent simplicity and low cost, have been procedures based on the differential solubility of polymeric glutenin and the monomeric proteins in various solvents and pHs. Some of the more frequently used methods, which

also vary in the type of starting material used (i.e. flour, dough or gluten), include modified Osborne sequential fractionation (Chen and Bushuk 1970, Bietz and Wall 1975), pH precipitation (Jones et al 1959, Orth and Bushuk 1973), and various other solvent fractionation approaches (MacRitchie 1978, Danno 1981, Kruger et al 1988, Burnouf and Bietz 1989). Some workers have combined different approaches to study particular fractions, e.g. modified Osborne fractionation followed by size-exclusion chromatography to separate gliadins from ethanol-soluble low molecular weight glutenin (Huebner and Bietz 1993), and size-exclusion chromatography followed by ion-exchange chromatography to isolate and purify glutenins (Lew et al 1992). Comparative analysis of some of these methods (Orth and Bushuk 1973, Khan and Bushuk 1979, Chakraborty and Khan 1988a), and overall results have indicated that the success of these techniques to produce consistent yields of relatively pure gliadin and glutenin fractions has been elusive. The problematic nature of separation of wheat proteins based on solubility has been reviewed (Miflin et al 1983).

A more direct approach to isolate polymeric glutenin is to avoid gluten formation entirely and first quantitatively remove the monomeric proteins by direct extraction of flour with non-reducing solvents such as dimethyl sulfoxide (Burnouf and Bietz 1989, Gupta and MacRitchie 1991) or 50% 1-propanol (Byers et al 1983, Singh et al 1991), thus leaving a starchy residue containing the polymeric glutenin which can be subsequently extracted under reducing conditions. While this type of procedure appears to be successful in obtaining relatively pure glutenin in essentially one step, it also results in the extraction of some soluble glutenin. The nature and amount of this soluble glutenin

which can be directly extracted from flour using non-reducing solvents has been largely ignored in the scientific literature.

While much useful information on the composition and functionality of wheat flour proteins has been accumulated, there still is no simple method for obtaining polymeric protein that is free of contamination by the monomeric proteins and *vice versa*. This study describes a new relatively straightforward procedure for preparing soluble and insoluble polymeric glutenin and monomeric proteins of high purity. The procedure is based on the differential solubility of these proteins in aqueous 50% and 70% 1-propanol. The procedure was tested by fractionating the proteins of flour milled from two bread wheat cultivars of widely different strength.

Materials and Methods

Wheat Cultivars

Samples of two Canadian wheat cultivars, Glenlea and Katepwa, were selected for this study. Glenlea is a cultivar of the Canada Western Extra Strong Red Spring wheat class which is noted for its extra strong dough mixing characteristics. Katepwa is a Canada Western Red Spring wheat cultivar. The wheats were milled on a Buhler experimental mill into straight grade flour. The protein ($N \times 5.7$, 14% m.b.) contents of the flours were 13.7% and 13.3% for Glenlea and Katepwa, respectively.

Extraction of Wheat Proteins Using an Osborne-type Fractionation

Glenlea and Katepwa flours (2.5 g) were extracted at room temperature (23 ± 1 °C)

with 12.5 ml of 0.5 M NaCl solution in a 30 ml centrifuge tube for 1 hr with brief vortexing every 15 min. Each suspension was centrifuged for 10 min at 3000*g*, and the supernatant was collected. To remove residual salt, the residue was washed two times with 7.5 and 5.0 ml of deionized and distilled water for 2 min each, followed by centrifugation. The three supernatants were pooled. The remaining residue was then extracted three times with 12.5, 7.5, and 5.0 ml of 50%(v/v) 1-propanol (BDH, HiPerSolv HPLC grade) for 1, 0.5, 0.5 hr, respectively, with brief vortexing every 15 min. The three 50% 1-propanol supernatants were pooled. Aliquots of each fraction and freeze-dried residue were analyzed by acid-polyacrylamide gel electrophoresis (A-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Fractionation and Quantification of Wheat Proteins Based on Their Solubility in 1-propanol Solutions

Flour samples (2.5 g) were sequentially extracted at room temperature with 12.5, 7.5, and 5.0 ml of 50% 1-propanol for 1.0, 0.5, and 0.5 hr, respectively, with brief vortexing every 15 min. Following centrifugation (10,000*g*, 10 min), and pooling of the three supernatants, hereafter referred to as the 50% 1-propanol soluble (50PS) fraction, the protein content was determined by micro-Kjeldahl method (AACC 1983). As with other fractions described below, samples for Kjeldahl analysis were prepared in triplicate. The residue was freeze dried and its protein content was determined likewise.

Glutenin was precipitated from a 5.0 ml aliquot of the pooled 50PS fraction by the addition of 3.4 ml 1-propanol to bring the final 1-propanol concentration to 70% (v/v).

The mixture was vortexed and allowed to stand at room temperature for 1 hr. After centrifugation (20,000*g*, 10 min), a 1.0 ml aliquot of the supernatant was used for protein determination by the micro-Kjeldahl method. The concentration of protein in the 70% 1-propanol precipitate was calculated by difference. Three protein fractions were obtained through the fractionation procedure as described above, *viz*. 50% 1-propanol insoluble residue (50PI), and two subfractions of the 50PS fraction: 70% 1-propanol insoluble (70PI) and soluble (70PS) material.

To analyze the protein composition of the 70PI and 70PS fractions by electrophoresis or RP-HPLC, the scale of the 70% 1-propanol precipitation procedure was reduced using 0.5 ml of the 50PS fraction. After centrifugation (15,000*g*, 10 min), the supernatant and precipitate were evaporated to dryness in a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY).

A-PAGE

The conditions of A-PAGE were as described by Sapirstein and Bushuk (1985). In order to check the presence of monomeric proteins of various fractions, a short run (about 100 min) procedure was employed to retain the gliadins and the higher mobility albumins and globulins within the gel.

The 50PS fraction (0.5 ml) was mixed with 0.25 ml of 0.25% (w/v) aluminum lactate buffer (pH 3.1) solution containing 40% (w/v) sucrose and 0.5% (w/v) methyl green dye (extract dilution solution). The dried 70PI and 70PS fractions were redissolved in 0.5 ml of 50% 1-propanol and mixed with 0.25 ml of extract dilution solution. To

check 50PI glutenin for the presence of monomeric proteins, 50 mg of freeze-dried sample was extracted with 0.2 ml 50% 1-propanol at room temperature for 1 hr with intermittent vortexing. After centrifugation (15,000g, 10 min), an aliquot of 0.1 ml of clear supernatant was mixed with 0.05 ml of extract dilution solution.

SDS-PAGE

SDS-PAGE was carried out according to Ng and Bushuk (1987) with some modifications. The stacking and separating acrylamide gel concentrations were 3.5 and 14%, respectively. Electrophoresis was carried out for 4 hr at 25 mA. Dried 70PS and 70PI fractions (from 0.5 ml of the 50PS fraction) were redissolved in 1.0 ml of buffer solution, pH 6.8, containing 0.063 M Tris-HCl, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.01% (w/v) pyronin Y (SDS extraction buffer). The 50PS fraction (0.5 ml) was mixed with 0.5 ml of SDS extraction buffer. The 50PI fraction (50 mg) was extracted with 0.5 ml of 50% 1-propanol containing 1% (w/v) dithiothreitol (DTT) and an aliquot (0.3 ml) was mixed with 0.3 ml of SDS extraction buffer.

RP-HPLC

Samples were analyzed using a Hewlett-Packard 1090M liquid chromatograph incorporating a DR5 solvent delivery system, autosampler, and heated column compartment maintained at 50°C for analyses. A Zorbax 300 SB-C8 (Rockland Technologies, Inc., Newport, DE) column (300 Å pore size, 5 μm particle size, 15 cm

×4.6 mm i.d.) was used in conjunction with a Zorbax 300 SB-C8 cartridge guard column (1.25 cm × 4 mm i.d.). Solvents for RP-HPLC were A) distilled water, deionized, purified, filtered (0.2 μm) using a Milli-Q system and deaerated, and B) acetonitrile (ACN, HPLC grade, 0.45 μm filtered, Burdick and Jackson). Both solvents contained 0.1% (v/v) trifluoroacetic acid (HPLC grade, Sigma). Solvents were continuously and slowly sparged with helium during analyses. Solvent flow rate was maintained at 1.0 ml/min. After sample injection and an initial 3 min isocratic condition at 23% solvent B, proteins were eluted in a 82 min linear gradient from 23-44% solvent B. The column was equilibrated at 23% solvent B for 8 min between runs. The column eluent was monitored at 214 nm using a Hewlett Packard 1090 diode array detector-series II incorporating a 6-mm path length, 8 μl flow cell and 4 nm slit assembly. Control of the chromatograph and data quantitation was provided by Hewlett-Packard HPLC Chemstation DOS series software implemented on a 486/50 MHz personal computer.

Sample Preparation for RP-HPLC

The method used to prepare samples for RP-HPLC was partly adapted from procedures described by Singh et al (1991) and Marchylo et al (1989). Four stock solutions are required: (A) 50% (v/v) 1-propanol, (B) 0.08 M Tris-HCl, pH 7.5 containing 50% (v/v) 1-propanol, (C) solution "B" containing 1% (w/v) DTT, (D) solution "B" containing 14% (v/v) 4-vinylpyridine. Control samples for RP-HPLC comprised reduced and alkylated solution "A"-insoluble flour protein, i.e. mainly glutenin which was prepared as follows. Flour (50 mg) was extracted twice with 1 ml

of 50% (v/v) 1-propanol for 30 min at room temperature, with intermittent vortexing, and centrifuged for 3 min at 2200*g* in a Heraeus-Christ Biofuge A microcentrifuge. The residue was washed with 1 ml of 50% 1-propanol for approximately 1 min, centrifuged, and the supernatant was discarded. The remaining solvent was removed by careful aspiration using a Pasteur pipette. The residue, or alternatively 70PI from 0.5 ml 50PS, or freeze-dried 50PI fractions (50 mg), was reduced with 0.1 ml of freshly prepared solution "C" for 1 hr at 60°C. This was followed by alkylation with 0.1 ml of solution "D" at 60°C for 15 min just prior to RP-HPLC analysis. The samples were vortexed briefly during reduction and alkylation. Following alkylation, samples were centrifuged for five min at 15,000*g*, and supernatants were carefully removed and syringe-filtered (0.45 μm Millex HV) into microvials which were subsequently sealed for RP-HPLC analysis.

Collection of RP-HPLC Peaks for SDS-PAGE

To collect sufficient protein of RP-HPLC peaks, a multiple sample injection procedure was used (Marchylo and Kruger 1988). The chromatogram was divided into three regions based on retention times to separate ω-gliadins, high molecular weight glutenin subunits (HMW-GSs) and low molecular weight glutenin subunits (LMW-GSs). Peaks in each region were collected manually and evaporated to dryness in a Speed Vac Concentrator. Samples were redissolved in 200 μl of SDS extraction buffer and 20 μl Aliquots were analyzed by SDS-PAGE.

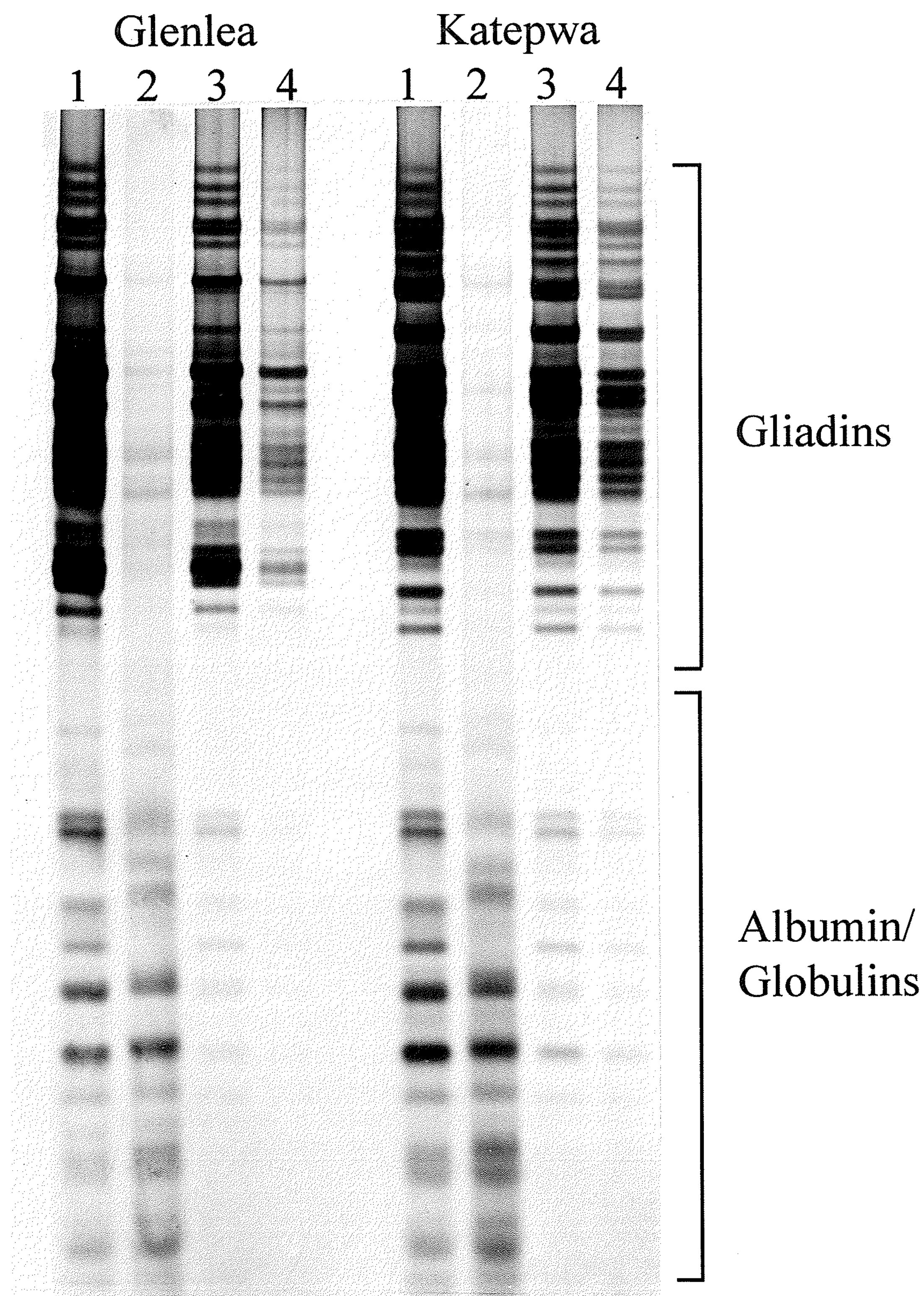
RESULTS

Composition of Osborne-type Wheat Protein Fractions

The modified Osborne protein fractionation scheme (Chen and Bushuk 1970) first extracts albumin and globulin proteins using a 0.5 M salt (NaCl) solution. Protein in the first residue which is extractable with aqueous alcohol has been termed gliadin, while the remaining residue has been widely referred to as glutenin which is partly soluble in dilute acid or dissociating agents. A-PAGE patterns of Osborne-type extracts (Fig. 3.1) shows that the 50% 1-propanol soluble fraction (lane 3) and the insoluble residue (lane 4) were contaminated with albumin and globulin proteins. It is particularly noteworthy that the 50% 1-propanol insoluble fraction (lane 4), which we previously assumed to contain only glutenin protein, was highly contaminated with gliadins. Furthermore there appeared to be a noticeably smaller amount of gliadins in the residue fraction of Glenlea compared to Katepwa. Similar results have been obtained in a related publication (Dupuis et al 1996) involving different samples of Katepwa and Glenlea wheats. In that study, it was found that the Osborne acetic acid-soluble protein fraction contained substantial quantities of gliadin proteins; accounting for 36% and 55% of the fraction (2.0% and 4.6% of total flour protein) in Glenlea and Katepwa, respectively.

Using an analogous sequential fractionation procedure, Singh et al (1991) reported some difficulty in resuspending the residue (i.e. glutenins) remaining after the initial salt and propanol extractions. The difficulty in the extraction of gliadins by aqueous alcohol after salt extraction may be due to the partial formation of dough in the centrifuged salt-insoluble residue. Furthermore, the salt in the first extract may induce aggregation of the

Fig. 3.1. Polyacrylamide gel electrophoresis at pH 3.1 of Osborne-type wheat protein fractions. Lane 1, control (flour proteins directly extracted in 50% 1-propanol); lane 2, flour proteins soluble in 0.5 M NaCl; lane 3, residue of NaCl extraction soluble in 50% 1-propanol; lane 4, 50% 1-propanol insoluble residue.


Glenlea
Katepwa
1 2 3 4
1 2 3 4
Gliadins
Albumin/
Globulins

gliadins with glutenin and thereby decrease their subsequent solubility in aqueous alcohol. Conversely, the alcohol-soluble Osborne fraction, when examined by SDS-PAGE under reducing conditions, clearly revealed the presence of all the HMW glutenin subunits for samples of both Katepwa and Glenlea (results not shown).

Fractionation of Proteins into 50% 1-propanol Soluble and Insoluble Fractions

Because of the relative ineffectiveness of 0.5M salt solution to completely extract the albumin and globulin proteins from wheat flour, and the subsequent difficulties in extracting the gliadins from the residual polymeric glutenins using 50% 1-propanol, extraction sequence was reversed to examine the efficiency of the alcohol extraction, i.e. proteins were first extracted with 50% 1-propanol followed by 0.5 M NaCl extraction of the remaining residue. The A-PAGE patterns of the resulting fractions (Fig. 3.2) clearly showed that 50% 1-propanol appears to extract essentially all of the monomeric proteins in the Katepwa and Glenlea flours. Subsequent extracts of the alcohol-insoluble residue with either salt solution (Fig. 3.2, lane 2) or additional 1-propanol (Fig. 3.2, lane 3) failed to reveal any bands by A-PAGE. However, as HMW-GSs were clearly evident by SDS-PAGE of the reduced 50% 1-propanol soluble protein (Fig. 3.3, compare lanes 2 and 3), a substantial amount of polymeric glutenin was also extracted with the monomeric proteins in this fraction. The next step was to isolate the glutenin from the 50PS protein fraction.

One additional observation on the SDS-PAGE separation of the reduced propanol insoluble residue is in regard to presence in Glenlea (Fig. 3.3, lane 3) of a single

Fig. 3.2. Polyacrylamide gel electrophoresis at pH 3.1 of (lane 1) 50% 1-propanol soluble flour proteins; lane 2, residue of 50% 1-propanol extract soluble in 0.5 M NaCl; lane 3, freeze-dried residue re-extracted with 50% 1-propanol.

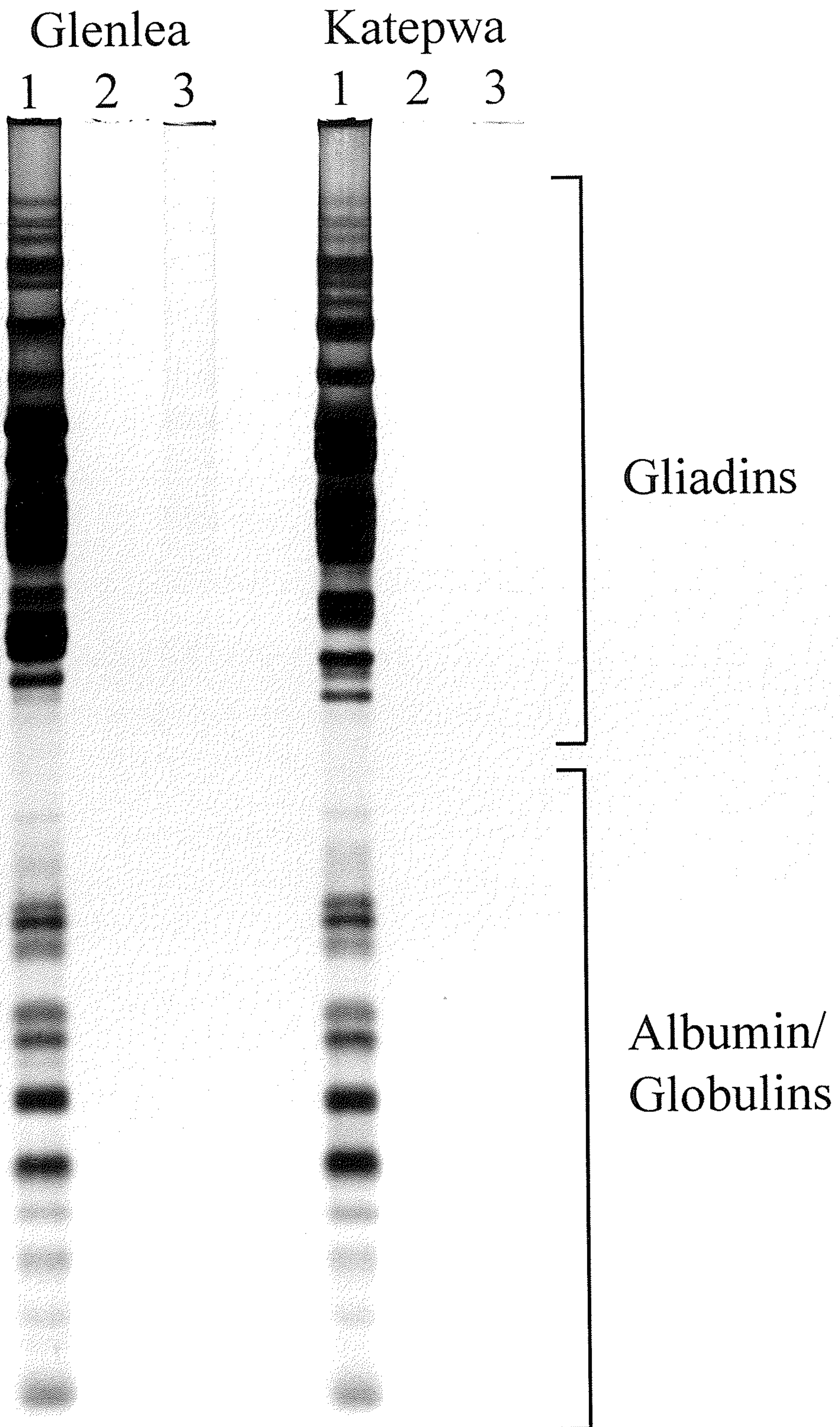
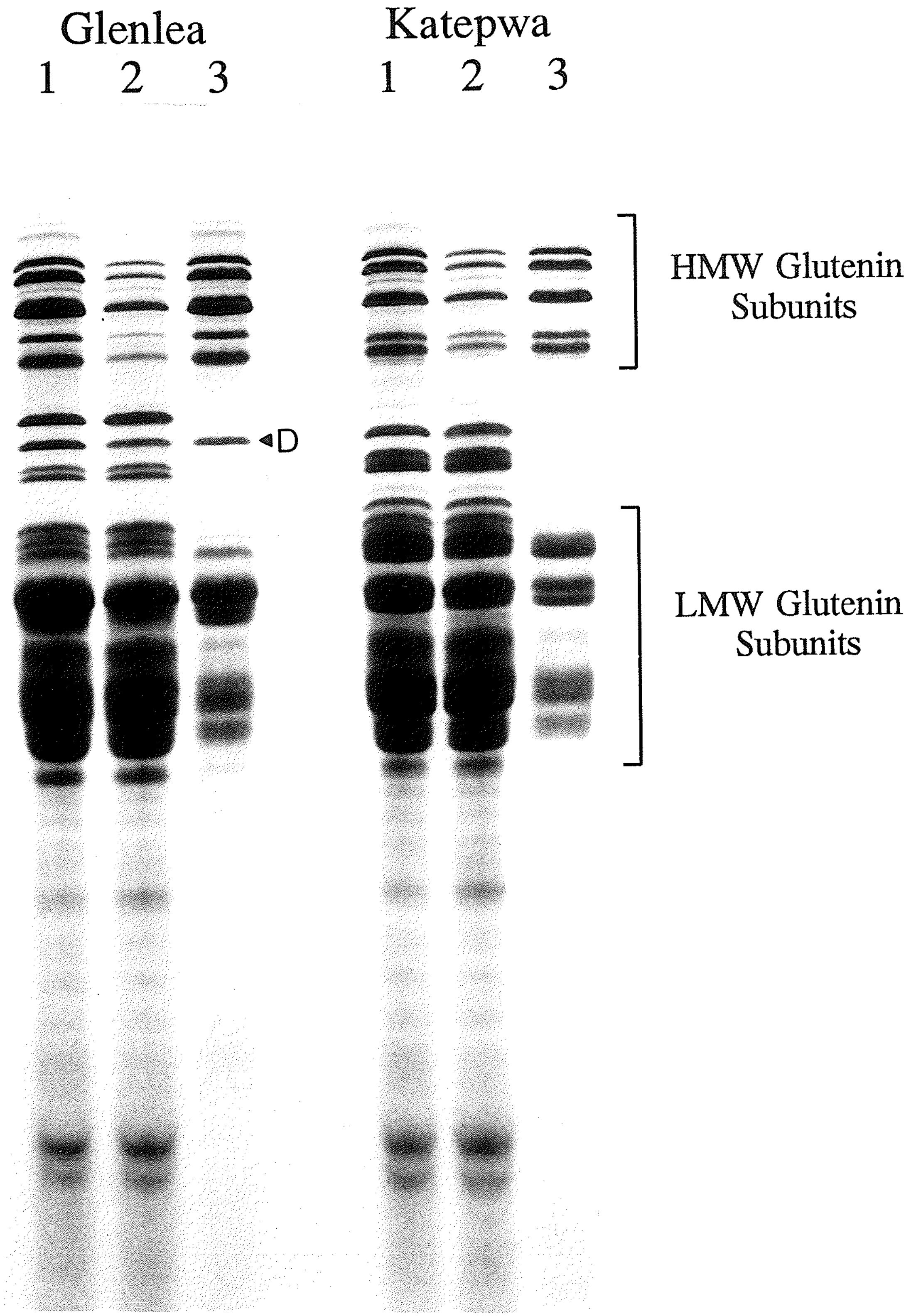

Glenlea
1 2 3
Katepwa
1 2 3
Gliadins
Albumin/
Globulins

Fig. 3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of (lane 1) reduced total flour proteins; lane 2, reduced 50% 1-propanol soluble flour proteins; lane 3, reduced 50% 1-propanol insoluble residue. D-glutenin subunit in Glenlea (lane 3) marked by 'D'.


Glenlea
1 2 3
Katepwa
1 2 3
D
HMW Glutenin Subunits
LMW Glutenin Subunits

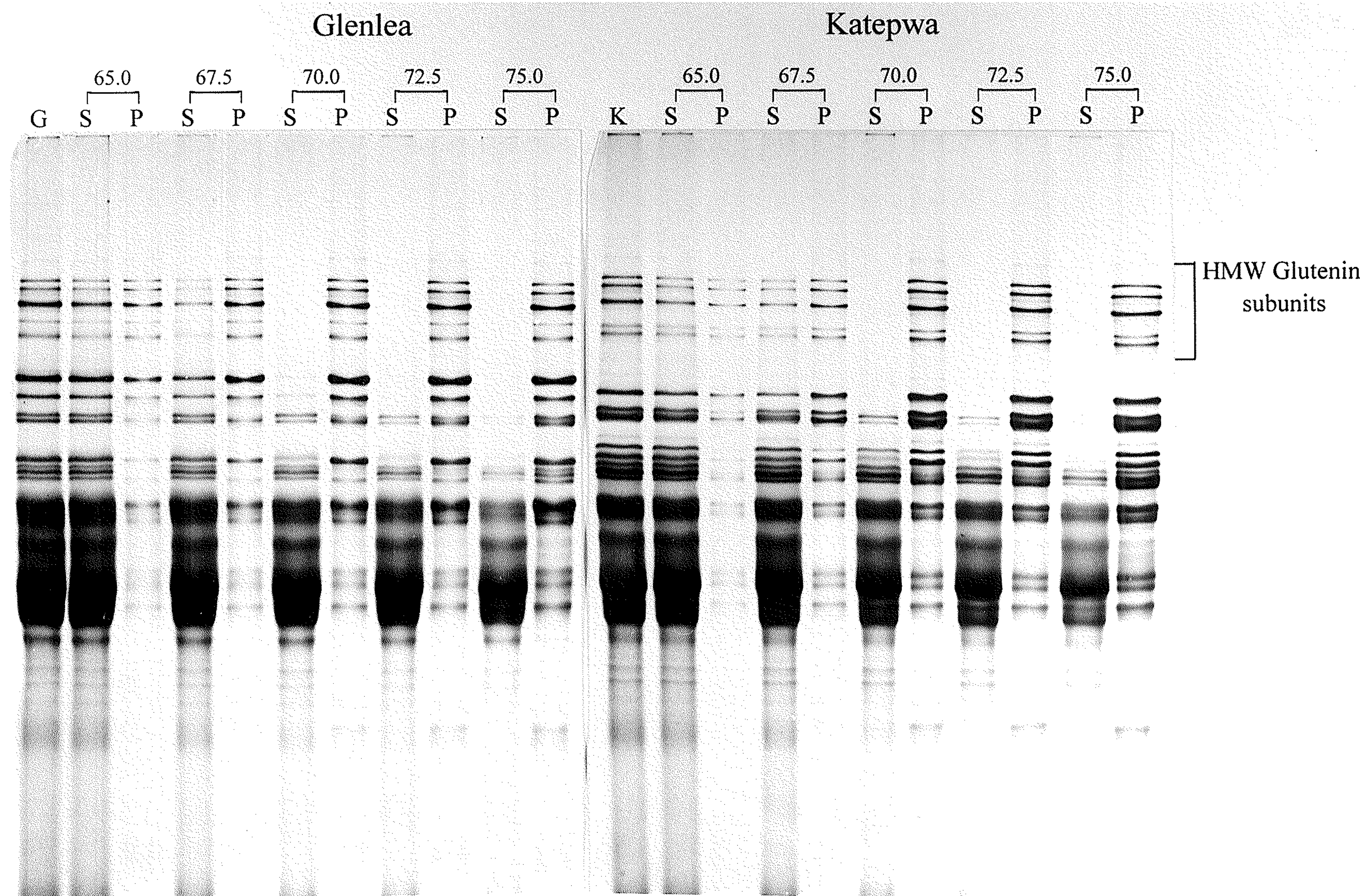
band in a region between the HMW- and LMW-GSs. The relative mobility of this band in the 50% 1-propanol insoluble residue separated in the presence of a reducing agent, and its complete absence in 50% 1-propanol extracts of flour separated by SDS-PAGE under non-reducing conditions (result not shown) are consistent with its identity as a D-glutenin subunit (Jackson et al 1983). The presence and absence of such subunits, which are coded by genes on the short arm of group 1 chromosomes, has been indirectly associated with negative and positive contributions to mixing and baking quality, respectively (Masci et al 1993). These workers also pointed out that this may be the case only for varieties without excessive strength and elasticity. As Glenlea can be classified as an extra strong dough mixing variety, the significance of its D-glutenin subunit in relation to technological quality is unknown.

Isolation of Glutenin from 50PS Fraction

Preliminary experiments showed that an increasing amount of protein could be precipitated from the 50PS fraction by raising the 1-propanol concentration above 50%. Therefore, the effect of the 1-propanol concentration on the type and amount of precipitated proteins was analyzed. In these experiments, pure 1-propanol was added to freshly prepared 50PS extracts to achieve concentrations of 65.0, 67.5, 70.0, 72.5 and 75.0%. After a 1 h rest at room temperature, followed by centrifugation (15,000*g*, 10 min), the respective residues and supernatants were evaporated to dryness, and analyzed by SDS-PAGE and A-PAGE.

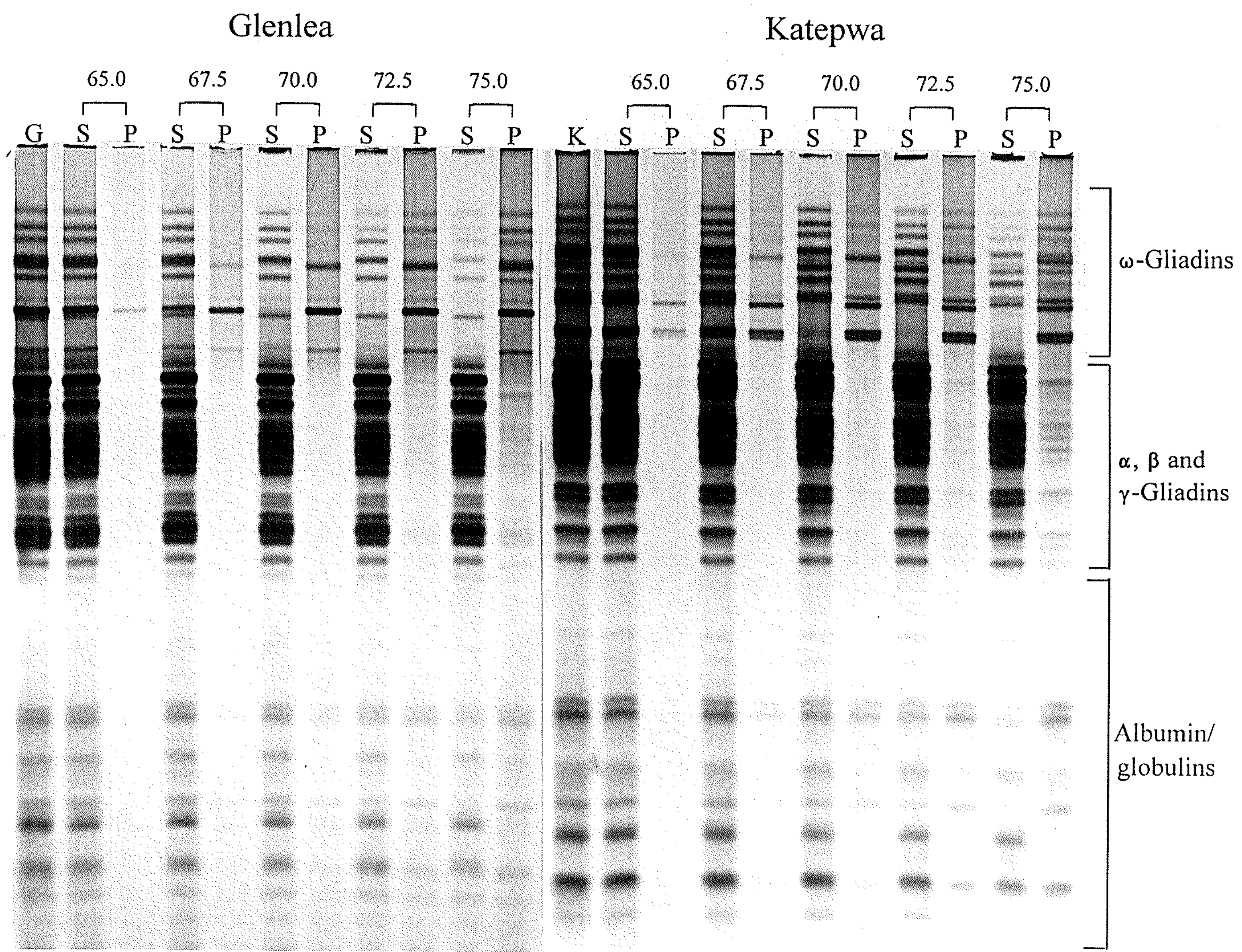
SDS-PAGE results (Fig. 3.4) showed that 1-propanol concentrations below 70%

Fig. 3.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reduced supernatants (S) and precipitates (P) of 50% 1-propanol soluble flour proteins obtained by adding 1-propanol to concentrations of 65.0, 67.5, 70.0, 72.5 and 75.0%. Lanes denoted as G and K are reduced 50% 1-propanol soluble proteins of Glenlea and Katepwa, respectively.



Glenlea
Katepwa
65.0
67.5
70.0
72.5
75.0
65.0
67.5
70.0
72.5
75.0
G S P S P S P S P S P
K S P S P S P S P S P
HMW Glutenin subunits

Fig. 3.5. Polyacrylamide gel electrophoresis at pH 3.1 of supernatants (S) and precipitates (P) of 50% 1-propanol soluble flour proteins obtained by adding 1-propanol to make various concentrations as in Fig. 3.4.


Glenlea
Katepwa
65.0
67.5
70.0
72.5
75.0
65.0
67.5
70.0
72.5
75.0
G S P S P S P S P S P
K S P S P S P S P S P
ω-Gliadins
α, β and
γ-Gliadins
Albumin/
globulins

left a significant amount of glutenin unprecipitated as evident by the presence of HMW-GS in the SDS-PAGE patterns of the supernatant fractions of both flours. In contrast, the 70PS supernatant protein contained no HMW-GS (Fig. 3.4), indicating that all the polymeric glutenin present in the 50PS fraction was precipitated at 70% aqueous 1-propanol. The effect of increasing the 1-propanol concentration above 70% was effectively shown by A-PAGE. The A-PAGE composition of the same fractions (Fig. 3.5) analyzed by SDS-PAGE showed that the 70% 1-propanol fractionation produced the greatest differentiation between the soluble and precipitated fractions. A small amount of monomeric proteins, mainly ω-gliadins and some albumins and globulins, co-precipitated with the polymeric glutenin in 70% 1-propanol. However, as the 1-propanol concentration increased to 75%, considerably more of the monomeric proteins was precipitated with glutenin. These results indicate that, based on visual examination of the electrophoretic results, 70% appears to be the optimum concentration of 1-propanol to precipitate the polymeric glutenin with the least contamination of monomeric proteins.

Composition of 1-propanol Soluble and Insoluble Fractions by Reversed-Phase High Performance Liquid Chromatography

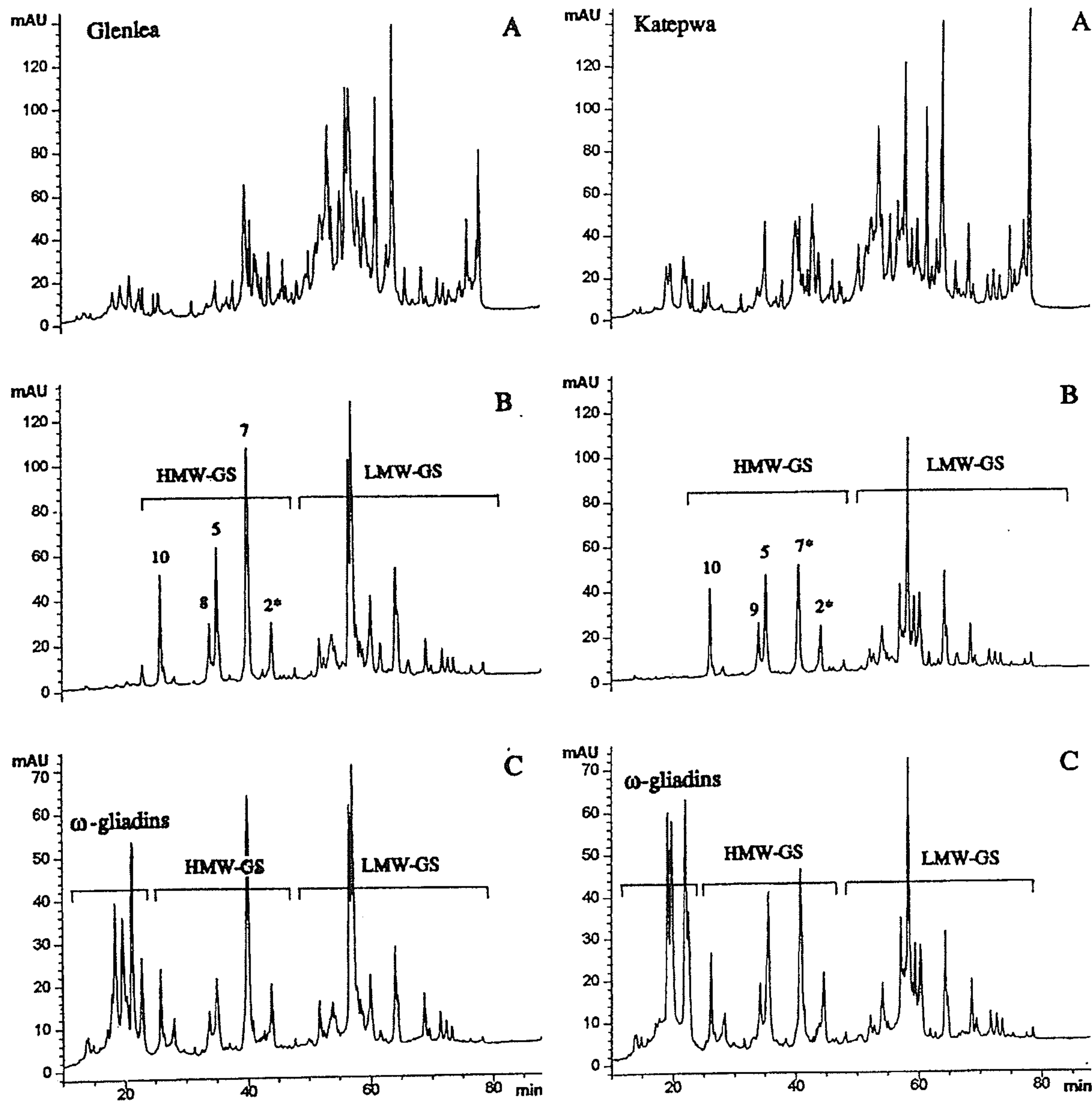
The highly complex and heterogeneous nature of wheat protein composition is well illustrated by RP-HPLC (Fig. 3.6). While the presence of glutenin subunits was not evident in the 50PS fraction chromatograms (Fig. 3.6A), they were clearly revealed upon precipitation of this fraction with 70% 1-propanol (Fig. 3.6C). Compared with the chromatograms of the 50PI glutenin fraction (Fig. 3.6B) which was essentially free of

monomeric proteins, the 70PI fraction (Fig. 3.6C) contained from three to four additional components which eluted earlier than the HMW-GSs. These peaks represent ω-gliadins (Bietz and Burnouf 1985, Kruger et al 1988, Marchylo et al 1989). Collection of these peaks (in addition to those of the HMW- and LMW-GSs) and analysis by SDS-PAGE (Fig. 3.7, lane 5) confirmed their identity with ω-gliadins given their electrophoretic mobility between the HMW-GSs and LMW-GSs (Fig. 3.7, lanes 1, 2 and 3). As RP-HPLC was effective in separating the 70PI glutenin from the co-precipitated monomeric proteins, the amount of glutenin that was soluble in 50% 1-propanol could be easily quantified (see below). The RP-HPLC result showed that the 50PI glutenin (Fig. 3.6B) and 70PI glutenin (Fig. 3.6C) have the same subunit composition. These two fractions were also essentially identical in the ratio of HMW to LMW glutenin subunit composition. For the 50PI fraction, the HMW-to-LMW ratios for Glenlea and Katepwa were $0.57 \pm .03$ and $0.47 \pm .02$, respectively, based on triplicated determinations. The corresponding ratios for 70PI glutenin were $0.54 \pm .04$ and $0.48 \pm .03$.

Quantification of Wheat Protein Fractions

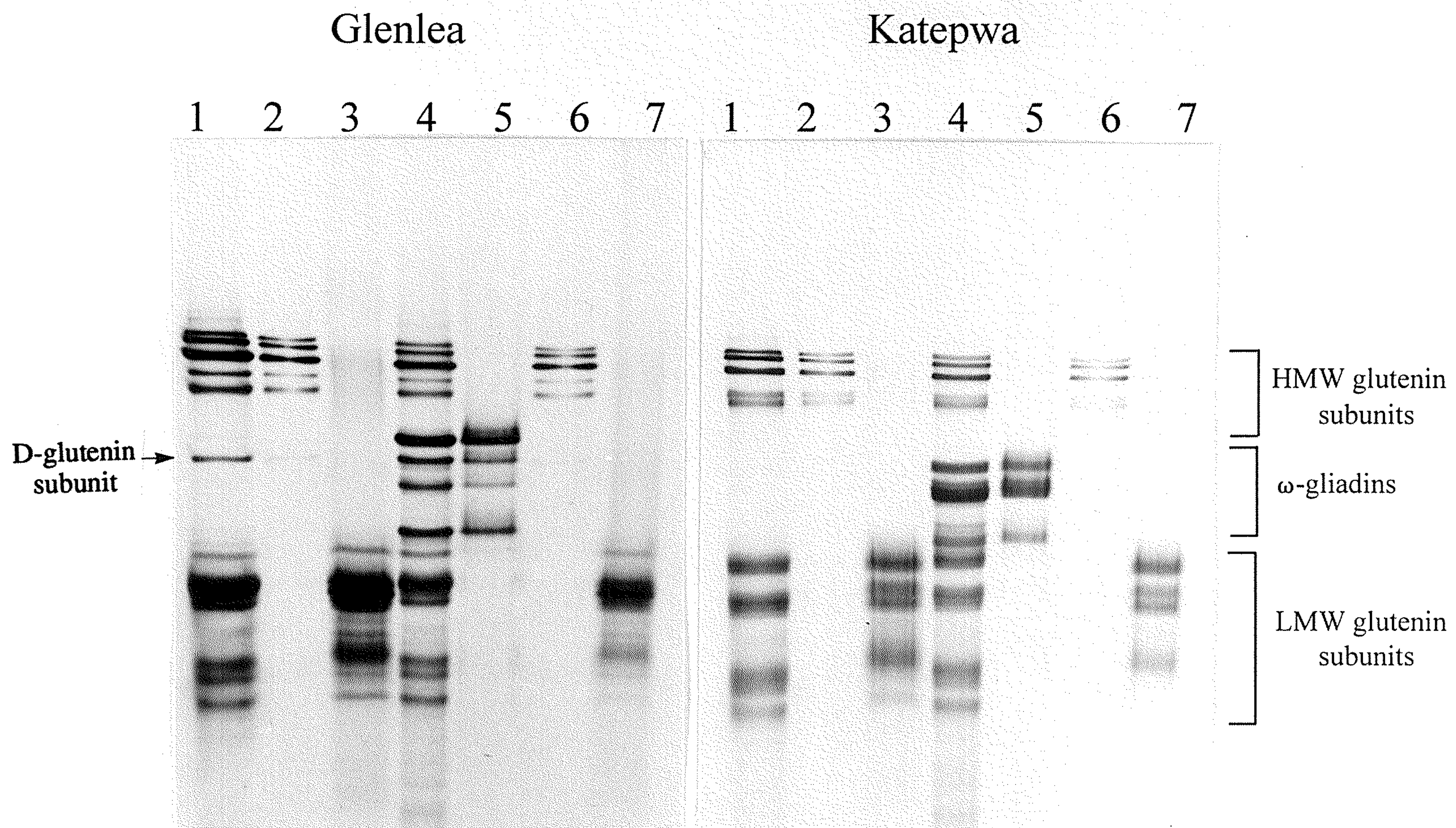
The relative amounts of the various 1-propanol soluble and insoluble fractions that were isolated are presented in Table 3.1. As the protein contents of both Glenlea and Katepwa flours were comparable, these data represent a close approximation of the difference in the absolute amount of glutenin and monomeric protein content in the two wheats. We found that approximately 50% of the total flour protein in both cultivars comprised glutenin; Glenlea had a moderately higher content of glutenin than Katepwa

Fig. 3.6. Reversed-phase high performance liquid chromatograms of reduced and alkylated flour proteins of Glenlea and Katepwa wheats. A, 50% 1-propanol soluble fractions; B, 50% 1-propanol insoluble residue. C, precipitate of 50% 1-propanol soluble fraction obtained by increasing the 1-propanol concentration to 70% (70PI fraction in text).



Glenlea
Katepwa
A
B
C
mAU
HMW-GS
LMW-GS
10
5
7
8
2*
9
7*
ω-gliadins
min

Fig. 3.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reduced Glenlea and Katepwa wheat flour proteins: Lane 1, 50% 1-propanol insoluble residue; Lanes 2 and 3, collected peaks by RP-HPLC corresponding to HMW- and LMW-glutenin subunits, respectively of Fig. 3.6B; Lane 4, precipitate of 50% 1-propanol soluble fraction obtained by increasing the 1-propanol concentration to 70% (70PI fraction in text); Lanes 5, 6 and 7: collected RP-HPLC peaks of 70PI fraction corresponding to regions of ω-gliadins, HMW- and LMW-glutenin subunits, respectively in Fig. 3.6C.



Glenlea
Katepwa
1 2 3 4 5 6 7
1 2 3 4 5 6 7
D-glutenin subunit
HMW glutenin subunits
ω-gliadins
LMW glutenin subunits

TABLE 3.1

Amount of 1-propanol Fractionated Protein in Katepwa and Glenlea as a Percentage of Total Flour Protein[a]

Cultivar	Monomeric Protein[b]	70PI Glutenin[c]	50PI Glutenin[d]	Total Glutenin	70PI in Total Glutenin
Glenlea	47.9±1.4	9.4±0.4	42.7±1.0	52.1±0.8	18.0±0.6
Katepwa	52.0±1.6	12.8±0.2	35.2±1.2	48.0±0.7	26.6±0.5

[a] Means of triplicate determinations ± standard deviations.

[b] 50% 1-propanol soluble protein less 70PI glutenin.

[c] Glutenin in 50% 1-propanol soluble fraction precipitated in 70% 1-propanol; values corrected according to RP-HPLC quantitation to exclude ω-gliadins; determined by difference.

[d] 50% 1-propanol insoluble glutenin.

(52% vs. 48%). For the 50PI glutenin, the difference increased to 7.5 percentage points, likewise in favor of Glenlea. Glenlea had approximately 21% more 50% 1-propanol insoluble glutenin compared with Katepwa, and 27% less glutenin that was extractable in the 50PS fraction (subsequently precipitated by aqueous 70% 1-propanol). About 18% of the Glenlea glutenin and 27% of the Katepwa glutenin were soluble in 50% 1-propanol extracts of flour. This quantity of "soluble" glutenin does not include the ω-gliadins which coprecipitate in the 70PI fraction (Fig. 3.6C). The amounts of these ω-gliadins, as a proportion of total protein in the 70PI fraction were comparable at 30.3 ± 1.4 and $32.8\% \pm .4\%$, for Glenlea and Katepwa, respectively.

The results presented above indicate that the main difference in the flour protein of the two wheat cultivars is in the relative quantities of the two types of polymeric glutenin that were isolated, soluble and insoluble in aqueous 50% 1-propanol. Accordingly, the ratio of 50PI to 70PI glutenin (Table 3.1) as a potential index of functionality of the flour protein for breadmaking was evaluated. The parameter so obtained will be referred to as the glutenin solubility index (GSI). The GSIs for Glenlea and Katepwa were 4.5 and 2.8, respectively. As Glenlea is known to be much stronger than Katepwa, both the magnitude and direction of difference in GSI values seem plausible. The mixograph dough development times (a commonly used index of flour strength) for the two wheats, $4.7 \pm .1$ and $2.9 \pm .02$ min for Glenlea and Katepwa, respectively, were directly proportional to the GSI values. The protein fractionation procedure reported in this study requires evaluation with many more wheat samples of diverse genotype and breadmaking quality before any robust conclusions can be drawn

regarding the direct relationship between GSI and dough mixing properties. However, the similarity between GSI and mixing time values obtained in this study is compelling, and points to the importance of obtaining an accurate quantitation of glutenin in flour, and the potential practical benefits of separating total glutenin into soluble and insoluble fractions.

One further observation regarding the 50PI glutenin fraction follows in relation to its quantification by RP-HPLC after reduction and alkylation. In this case, the magnitude of the difference between Glenlea and Katepwa was substantially higher than that obtained by Kjeldahl protein analysis of the 50PI starchy residue. Replicated quantitation of the integrated areas of the chromatograms of the 50PI fraction (as in Fig. 3.6B) showed that Glenlea contained approximately 42% more glutenin compared to Katepwa. It appears that the quantification accuracy of our relative protein determination by RP-HPLC may be suspect. In general, the quantitation accuracy by HPLC, when polymeric glutenin is involved, depends on many factors. Among the more important of these are the requirements for complete protein extraction (reduction), efficient and homogeneous filtering of the reduced and alkylated glutenin subunits, and complete elution of the glutenin subunits in the aqueous ACN gradient. Preliminary investigation of the first of these factors yielded an interesting result. Following the standard reduction and alkylation steps involving the 50PI protein, repeated extractions were carried out on the remaining residue with the 50% 1-propanol/1% DTT reducing solution "C". Subsequently, the dried residue was reduced with an aqueous solution of 2% SDS containing 1% DTT, thus essentially replacing 1-propanol with SDS in the reducing

solvent solution. SDS-PAGE (see Chapter 4) of the resulting extracts of both Katepwa and Glenlea samples revealed a substantial number of protein bands of varying intensity. The bands represented some, but not all of the HMW- and LMW-GS, as well as some novel subunits of unknown identity. Based on a subjective assessment of band staining intensity, there also appeared to be a greater quantity of protein in the Katepwa sample than in the Glenlea sample, which is consistent with the RP-HPLC integration results that were obtained. This indicates that a significant amount of polymeric protein is insoluble in 50% 1-propanol solutions containing reducing agent, and that the quantity of this residue glutenin was apparently not related to breadmaking quality. This result was further investigated in Chapter 4.

Discussion

The challenges in obtaining a sharp and accurate separation of polymeric and monomeric proteins of wheat flour using differential solubility techniques have been known by cereal chemists for a long time (Osborne 1907). Cross-contamination of the major wheat endosperm protein classes, i.e. monomeric proteins in glutenin preparations, and polymeric glutenin in gliadin preparations, represents the main problem in preparing relatively pure protein fractions for analysis and study.

While overlapping solubility of highly complex and heterogenous wheat protein fractions may partly explain the difficulties encountered, results reported in the scientific literature suggest that a principal factor contributing to cross contamination of some wheat protein fractions is the non-covalent chemical interactions that exist in the flour

or are induced by the solvents added. These interactions are likely increased in procedures where dough or gluten replaces flour as the starting material for protein fractionation. Also, it appears that interactions can be accentuated by flour defatting at the outset of the fractionation used in many studies; this results in the insolubilization of some albumins and globulins (Miflin et al 1980, Byers et al 1983).

Additional aggregation of monomeric and polymeric proteins may be induced by centrifugation used for separating the solubles. Centrifugation of protein-starch dispersions, as in Osborne type fractionations which begin by water or salt solution extraction of flour, concentrates monomeric and polymeric proteins in the insoluble residues. It seems plausible under these preparative conditions that non-covalent interactions between monomeric proteins and polymeric glutenin would be considerable. Moreover, in the modified Osborne procedure of Chen and Bushuk (1970), a flour sample is extracted initially in a 0.5 M salt solution to specifically accentuate the insolubility of the gliadins (which are quite soluble in deionized water), thus optimizing their separation from the albumins and globulins. Therefore, it should not be surprising to find considerable amounts of monomeric proteins, most notably the gliadins apparently insoluble in, or more correctly unextractable with, aqueous ethanol (Orth and Bushuk 1973, Bietz and Wall 1975, Dupuis et al 1996) or 1-propanol as shown in this study. As a result, a varying quantity of gliadins, an amount which seems to be genotype specific and inversely related to dough strength (Dupuis et al 1996), will then be extractable in the subsequent acetic acid fractionation of glutenin. Accordingly, the identity of acetic acid soluble flour protein (mixture of gliadin and glutenin) with glutenin

(Orth and Bushuk 1972) appears to be inaccurate.

In addition to difficulties in gliadin protein solubility created by using salt solutions to extract albumin and globulin proteins from flour, it appears that glutenin solubility is similarly affected. Polymeric glutenin, in particular, is known to be adversely affected by the presence of salt in the extraction solution (Huebner 1970, Hoseney et al 1969c, Kim and Bushuk 1995). Moreover the glutenin of stronger wheat varieties appears to be more sensitive to this effect than weaker varieties (Huebner 1970, Kim and Bushuk 1995). Accordingly, prior exposure of flour proteins to salt solutions, as in an Osborne-type fractionation, likely renders glutenin less soluble in acetic acid or similar solvents used for subsequent extraction and may even affect the subunit composition of extracted glutenin as was recently observed (Kim and Bushuk 1995).

This study has shown that much better flour protein fractionation is achievable by reversing the order of the Osborne solvents by using aqueous alcohol in the first step to remove monomeric proteins that would otherwise interact and aggregate with polymeric glutenin if salt solution is used first. While it is possible to fractionate monomeric flour proteins and polymeric glutenin according to their differential solubility in 50% 1-propanol, this separation is not complete; a significant quantity of polymeric glutenin, an amount which appears to be genotype specific (~18 and 26% of total glutenin in Glenlea and Katepwa, respectively), is extracted in this solvent. However, the glutenin soluble in 50PS can be easily separated from the monomeric proteins by selective precipitation in 70% 1-propanol. The supernatant of this fractionation contains albumins, globulins, and most of the gliadin proteins.

It was interesting to note that the selective precipitation of 70PI glutenin also resulted in the co-precipitation of a considerable amount of some ω-gliadins. The 70PI ω-gliadins, as quantified by RP-HPLC, represented almost one-third of the 70PI glutenin protein fraction. These ω-gliadins were distinguished from the other ω-gliadins in both Glenlea and Katepwa wheats by their relatively higher electrophoretic mobility in A-PAGE. On this basis it would appear that these ω-gliadins are coded by genes at the *Gli-A1* or *Gli-B1* loci as the slow-moving ω-gliadins are known to be coded by genes on the short arm of chromosome 1D (Shepherd 1968, Wrigley and Shepherd 1973, Sozinov and Poperelya 1982). The significance of this finding in regard to glutenin functionality is unclear. The reasons for the similarity in solubility of the 70PI glutenin and some ω-gliadins, as well as the possibility of a specific interaction between these proteins, remain to be investigated.

Conclusions

A new method for fractionation of monomeric and polymeric proteins of wheat flour has been developed. Proteins were first separated into 50% 1-propanol soluble and insoluble fractions. The 50PI protein was essentially free of monomeric proteins and comprised mainly glutenin, while 50PS protein was a mixture of monomeric proteins and polymeric glutenin. Polymeric glutenin in 50PS protein was isolated under non-reducing conditions by precipitation with 1-propanol to a concentration of 70%. The RP-HPLC and SDS-PAGE results showed that the 50PS and 70PI glutenin had the same subunit composition, and similar HMW to LMW subunit ratio. Thus we found no qualitative

difference between the soluble and insoluble polymeric fractions. On the other hand, quantitative differences were substantial. The proportion of total polymeric glutenin that was soluble in aqueous 1-propanol was much less in Glenlea wheat than Katepwa. Glenlea also contained significantly more 50PI glutenin. These results, and in particular the ratio of 50PI to 70PI glutenin, are consistent with the relative breadmaking quality characteristics of the two cultivars used in this study. The precise reason for the difference in solubility of the polymeric glutenin in aqueous 1-propanol remains to be elucidated, although a difference in glutenin molecular size is probably a principal factor. It seems likely that the very strong mixing characteristics of Glenlea flour derive from a greater percentage of larger-sized polymers than is present in Katepwa. The results obtained in this study confirm the importance of both the soluble and insoluble polymeric glutenin in determining dough strength (Orth and Bushuk 1972). The protein isolation procedure described herein should be useful for physicochemical characterization of soluble and insoluble glutenin fractions in further studies of the biochemical basis of breadmaking quality of wheat cultivars, as described in the following chapters.

Chapter 4

FRACTIONATION OF MONOMERIC PROTEINS, SOLUBLE AND INSOLUBLE GLUTENINS, AND RELATIONSHIPS TO MIXING AND BAKING PROPERTIES

Abstract

Wheat flour proteins of seven Canadian wheat cultivars of diverse breadmaking quality were first extracted with 50% 1-propanol. Essentially all monomeric proteins plus some glutenin were solubilized. The soluble glutenin was isolated by selective precipitation with 1-propanol to a concentration of 70%. The insoluble glutenin in the residue was then extracted three times with 50% 1-propanol containing 1% dithiothreitol (DTT) as a reducing reagent at 60°C. An appreciable amount of protein still remained in the final residue after above extractions. The protein fractions were quantified and characterized by electrophoresis. There was little variation among the seven cultivars in the amount of monomeric (48-52%) and residue (14-18%) protein fractions. The soluble- and insoluble- glutenin fractions showed greater intercultivar variation; 10-20% and 12-28% for the soluble and insoluble fractions, respectively. The intercultivar variation in these two fractions explained 80-90% of the variation in four dough strength parameters (mixograph dough development time and work input to peak, and extensigraph maximum resistance and area), and 70-80% of the variation in loaf volume. Interestingly, it was found that the *Glu-D1* high molecular weight glutenin subunits (HMW-GS) were dominant in the final residue compared with other HMW-GS.

Introduction

Since the first scientific classification of wheat proteins by Osborne (1907), cereal chemists have searched for a definitive explanation of the unique breadmaking ability of wheat flour on the basis of the molecular properties of its proteins. The uniqueness of wheat flour doughs derives from a unique combination of two physical properties: viscosity and elasticity. There is good scientific agreement that viscosity is generally associated with the monomeric protein components (mainly gliadins) of wheat endosperm, while elasticity is understood to be a function of polymeric fraction, i.e., glutenin (for review see Bushuk and MacRitchie 1989).

The basic distinction of functionality of gliadin and glutenin underscores the importance of a clean separation and accurate quantification of these two major components of wheat proteins. It has long been realized that distinction between solubility classes is not sharp because of the overlapping solubilities of proteins in highly heterogenous mixtures. In Osborne-type wheat protein fractions, for example, aqueous ethanol extracts a small amount of glutenin as well as most of the gliadin, and acetic acid-soluble glutenin can contain gliadins and other monomeric proteins (Orth and Bushuk 1973, Bietz and Wall 1975, Dupuis et al 1996). While glutenin free of monomeric proteins can be prepared by direct extraction of flour with dimethyl sulfoxide (Burnouf and Bietz 1989, Gupta and MacRitchie 1991) or 50% 1-propanol (Singh et al 1991), significant amounts of glutenin are also removed together with monomeric proteins. The nature and amount of the soluble glutenin, and its variability among genotypes, has been largely ignored in the literature. Presumably soluble glutenin

comprises polymers of smaller size compared to insoluble glutenin. In this regard, a size-based classification of glutenin by gel filtration chromatography (GFC) or size-exclusion high-performance liquid chromatography (SE-HPLC) was claimed to be superior to a solubility-based fractionation (Singh et al 1990a, Batey et al 1991). These studies also showed that considerable overlap exists in the size distribution of wheat proteins. In addition to the difficulty in solubilizing native glutenin, GFC and SE-HPLC lose their resolution for proteins above a certain molecular size, resulting in poor or no fractionation of glutenins.

Studies on the relative proportion of gliadin or glutenin in total flour protein and the molecular weight distribution (solubility) of glutenin have been an important part of the overall attempts to elucidate the relationship between protein structure and breadmaking quality. It would be a fair assessment, however, that progress has been hampered by the cross-contamination of the two major protein classes and poor separation of glutenins of different molecular sizes (for review see MacRitchie 1992).

In this chapter, previous research (Chapter 3) has been extended to meet the challenge of discriminative fractionation and quantification of wheat flour proteins using a relatively straightforward procedure based on the differential solubility of monomeric and polymeric proteins in different aqueous solutions of 1-propanol, yielding four relatively distinct protein fractions: one monomeric and three subfractions of polymeric protein. The protein composition of flours milled from seven wheat cultivars of diverse dough strength was examined and the relationships between the quantity of these fractions and dough properties and breadmaking quality were determined.

Materials and Methods

Flour Samples and Quality Tests

Seven wheat cultivars were chosen to represent a wide range of dough properties and baking potential (Table 4.1). Glenlea, Roblin and Katepwa are hard red spring wheat cultivars, with extra strong, very strong, and strong dough characteristics, respectively. Roblin and Katepwa are wheats belonging to the Canada Western Red Spring class, while Glenlea is a wheat cultivar of the Canada Western Extra Strong class. Biggar and AC Karma are cultivars of the Canada Prairie Spring wheat class. They exhibited strong and medium strong dough properties, respectively. SWS-52 and Harus are soft white spring and soft white winter wheats, respectively. Both have very weak dough properties. Glenlea, Roblin, Katepwa, Biggar and AC-Karma were milled to straight grade flour on a Buhler pneumatic laboratory mill. SWS-52 and Harus were similarly milled on an Allis Chalmers laboratory mill in which conditions were optimized for soft wheats. Dough mixing properties were measured using a 2-g direct drive computerized Mixograph (National Manufacturing, Lincoln, Nebraska). Extensigraph data were obtained according to AACC method 54-10 (AACC, 1983). The remix-to-peak baking test (Kilborn and Tipples, 1981) was used to evaluate the baking quality of the flours. This test evaluates baking potential of flour under optimized conditions of water absorption and dough development.

TABLE 4.1

Quality Characteristics of Flour Samples[a]

Cultivar[b]	Flour Protein[c] (%)	Mixograph DDT (min)	Mixograph WIP (%Tq*min)	Extensigraph Rmax (BU)	Extensigraph Ext (cm)	Extensigraph Area (cm^2)	Loaf Volume (ml)
Glenlea	13.7±0.1	5.3±0.1	211±13	1000±50	19.0±0.2	296±12	828±25
Roblin	13.6±0.0	3.4±0.1	141±4	560±14	23.0±0.7	196±7	1010±35
Katepwa	13.3±0.1	3.5±0.1	123±2	590±14	16.7±1.1	146±3	913±18
Biggar	12.5±0.1	3.7±0.0	141±3	720±7	22.5±0.4	234±6	970±7
AC Karma	10.9±0.2	2.6±0.1	69±2	390±11	18.0±1.1	108±6	603±25
SWS-52	9.5±0.0	1.9±0.0	42±1	245±3	17.5±0.3	61±5	423±4
Harus	9.5±0.1	2.0±0.1	30±1	170±2	16.0±0.6	41±2	355±7

[a] means of at least duplicates ± standard deviation
[b] ranking based on wheat class and mixing strength within class
[c] 14% m.b.

Abbreviations used: DDT = dough development time; WIP = work input to peak;
Rmax = maximum resistance; Ext = extensibility.

Solvents for Extraction of Flour Proteins

The following solutions were used for sequential extraction of flour proteins: A) 50% aqueous 1-propanol (BDH, HiPerSolv HPLC grade); B) solution "A" containing 1% dithiothreitol (DTT) (CALBIOCHEM); C) solution "A" adjusted to pH 7.5 with 0.08 M Tris-HCl buffer; D) solution "C" containing 1% DTT. Distilled water, deionized, purified, filtered (0.2 um) using a Milli-Q system was used to prepare all the solutions. Since protein determination by the Kjeldahl procedure cannot be performed in the presence of Tris-HCl buffer, solvent "B" was used for quantitation of proteins and solvent "D" for sample preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

Extraction and Quantitation of Flour Proteins

Flour (100.0 mg) was mixed with 1 ml of solvent "A" for 30 min at room temperature (23±1°C) in a microcentrifuge tube with intermittent vortexing, and centrifuged for 3 min at 2,200*g*, The supernatant was collected and the residue was extracted once more. The resulting residue was washed with 1 ml of solvent "A" for approximately 1 min and centrifuged for 3 min at 15,000*g*. The three supernatants were combined and analyzed for protein (N x 5.7) by the micro-Kjeldahl method 46-13 (AACC 1983). The residue remaining after the above extractions with solvent "A" was further extracted three times (1.0, 0.5, and 0.5 h, respectively) with 1 ml of solvent "B" at 60°C. The protein contents of the pooled supernatants and the final residue, were determined.

Sample Preparation for SDS-PAGE

The pooled supernatants extracted with solvent "A" as described above were evaporated to dryness in a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY). For SDS-PAGE, the dry material was dissolved in 1 ml of "SDS buffer" solution, comprising 0.063 M Tris-HCl (pH 6.8), containing 2% SDS, 1% DTT, 20% glycerol and 0.01% pyronine Y.

The insoluble residue after flour extraction with solvent "A" was first extracted with 1 ml of solvent "D" at 60°C for 1 h, then twice with the same solvent for 0.5 h with centrifugation (15,000*g*, 5 min) between extractions. The three supernatants were combined and evaporated to dryness. The dry material was dissolved in 1 ml of SDS buffer. The final residue (after extraction with solvent "D") was evaporated to dryness and extracted with 1 ml of SDS-PAGE buffer at room temperature for 1 h followed by centrifugation. The supernatant was used directly for SDS-PAGE.

Before extracting with SDS solution without reductant, the freshly prepared wet final residue was alkylated with 0.2 ml of 5% (w/v) N-ethylmaleimide (NEMI) in 50% 1-propanol at 60°C for 1 h followed by evaporation to dryness. To examine whether or not NEMI can efficiently inactivate DTT, 0.5 ml of 50% 1-propanol/1% DTT was mixed with 0.5 ml 50% 1-propanol/5% NEMI and incubated for 1 h at 60°C. Subsequently, 0.2 ml of this solution was used to extract 50 mg of flour; the extract was examined by SDS-PAGE to analyze for the presence or absence of HMW-GS.

SDS-PAGE was carried out according to Ng and Bushuk (1987) except that the stacking and separating gel concentrations were altered to 3.5 and 14.0%, respectively,

and separation was for 4 h at 25 mA.

Quantification of glutenin in 50% 1-propanol soluble fraction

Flour proteins were also fractionated according to the procedure developed in Chapter 3. In that procedure, 50% 1-propanol soluble glutenin was isolated by selective precipitation with 70% 1-propanol and quantified by RP-HPLC and micro-Kjeldahl analysis. The three protein fractions obtained were as followers: monomeric proteins, 50% 1-propanol soluble glutenin (precipitated by 70% 1-propanol) and 50% 1-propanol insoluble glutenin.

Statistical Analysis

Pearson correlation was performed using SAS 6.0 software (Cary, NC). The mean values for protein fraction parameters were based on triplicated extraction experiments.

Results and Discussion

Fractionation and Composition of Wheat Flour Proteins

Depending on the cultivar sample, between 57-70% of flour protein was extracted with 50% 1-propanol, and 12-28% more protein was solubilized by extraction of the residue with 50% 1-propanol under reducing conditions (Table 4.2). Despite the exhaustive extraction conditions, still 14-18% of the flour protein remained in the final residue.

TABLE 4.2

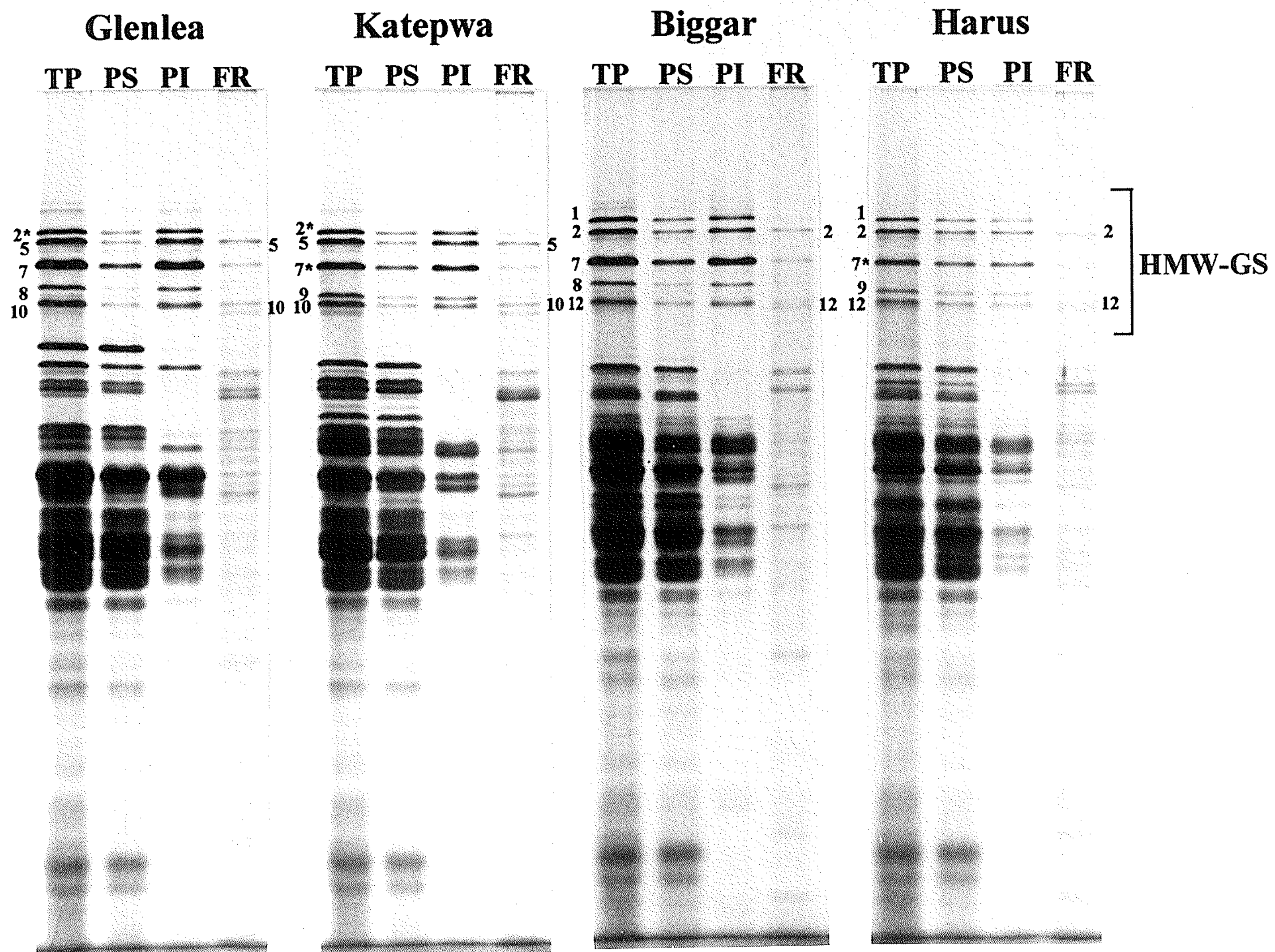
Percent Protein in Sequential Extracts from Different Wheat Flours[a,b]

Flour	50% 1-propanol	50% 1-propanol/ 1%DTT	Residue	Protein Recovery (%)
Glenlea	57.2 ± 0.8f	28.3 ± 0.4a	14.5 ± 0.2c	97.9 ± 1.3
Roblin	61.7 ± 0.3d	23.8 ± 0.1c	14.5 ± 0.1c	98.6 ± 0.5
Katepwa	62.9 ± 0.5c	19.9 ± 0.2d	17.2 ± 0.2ab	98.3 ± 0.8
Biggar	59.8 ± 0.3e	25.5 ± 0.3b	14.7 ± 0.4c	97.4 ± 0.6
AC Karma	64.8 ± 0.6b	20.5 ± 0.2d	14.7 ± 0.3c	96.8 ± 0.9
SWS-52	68.9 ± 0.4a	14.5 ± 0.6e	16.6 ± 0.8b	96.6 ± 1.1
Harus	69.6 ± 0.3a	12.6 ± 0.4f	17.8 ± 0.5a	97.1 ± 1.0
Mean	63.6	20.7	15.7	
CV[c]	7.2%	27.5%	9.1%	

[a] Percent of the sum of the proteins in all fractions.
[b] Means of three replicates ± standard deviation, means with the same letter are not significantly different (α = 0.05) between cultivars within a fraction.
[c] Coefficients of variation

Proteins extracted with 50% 1-propanol, 50% 1-propanol/1% DTT and the final residue were analyzed by SDS-PAGE (Fig. 4.1). For comparison, the total flour protein extracted with 2%SDS/1%DTT is also shown. The 50% 1-propanol efficiently extracts essentially all monomeric proteins (gliadins, albumins/globulins), plus some polymeric glutenins (see Chapter 3). Proteins subsequently extracted with 50% 1-propanol/1% DTT consisted of pure HMW-GS and LMW-GS (Fig. 4.1, lanes PI). These results are consistent with those reported previously (Byers et al 1983, Singh et al 1991). An appreciable amount of protein remained in the residue after sequential extraction with 50% 1-propanol under both non-reducing and reducing conditions. This was considered to be structural not storage protein in previous reports (Byers et al 1983, Kruger et al 1988). However, SDS-PAGE results (Fig. 4.1, lanes FR) clearly showed that the residue fraction contained some HMW-GS, i.e. storage proteins. Interestingly, the HMW-GS in the final residue were mainly those controlled by the *Glu-D1* locus (i.e., 5+10 in Glenlea and Katepwa, 2+12 in Biggar and Harus), although subunit 7 (*Glu-B1*) was the most abundant among HMW-GS in flour (Marchylo et al 1992). SDS-PAGE analysis of the final residue also indicated that very little LMW-GS was present. Most protein bands of this fraction (Fig. 4.1 lanes FR) with mobilities lower than HMW-GS are likely non-prolamin in nature (Gupta et al 1991b) as described further below.

Fig. 4.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins in sequential extracts of flours of Glenlea, Katepwa, Biggar and Harus. TP, total flour proteins extracted with SDS/dithiothreitol (DTT); PS, 50% 1-propanol soluble; PI, 50% 1-propanol/1% DTT soluble; FR, SDS-DTT soluble protein of the final residue. HMW-GS, high molecular weight glutenin subunits.

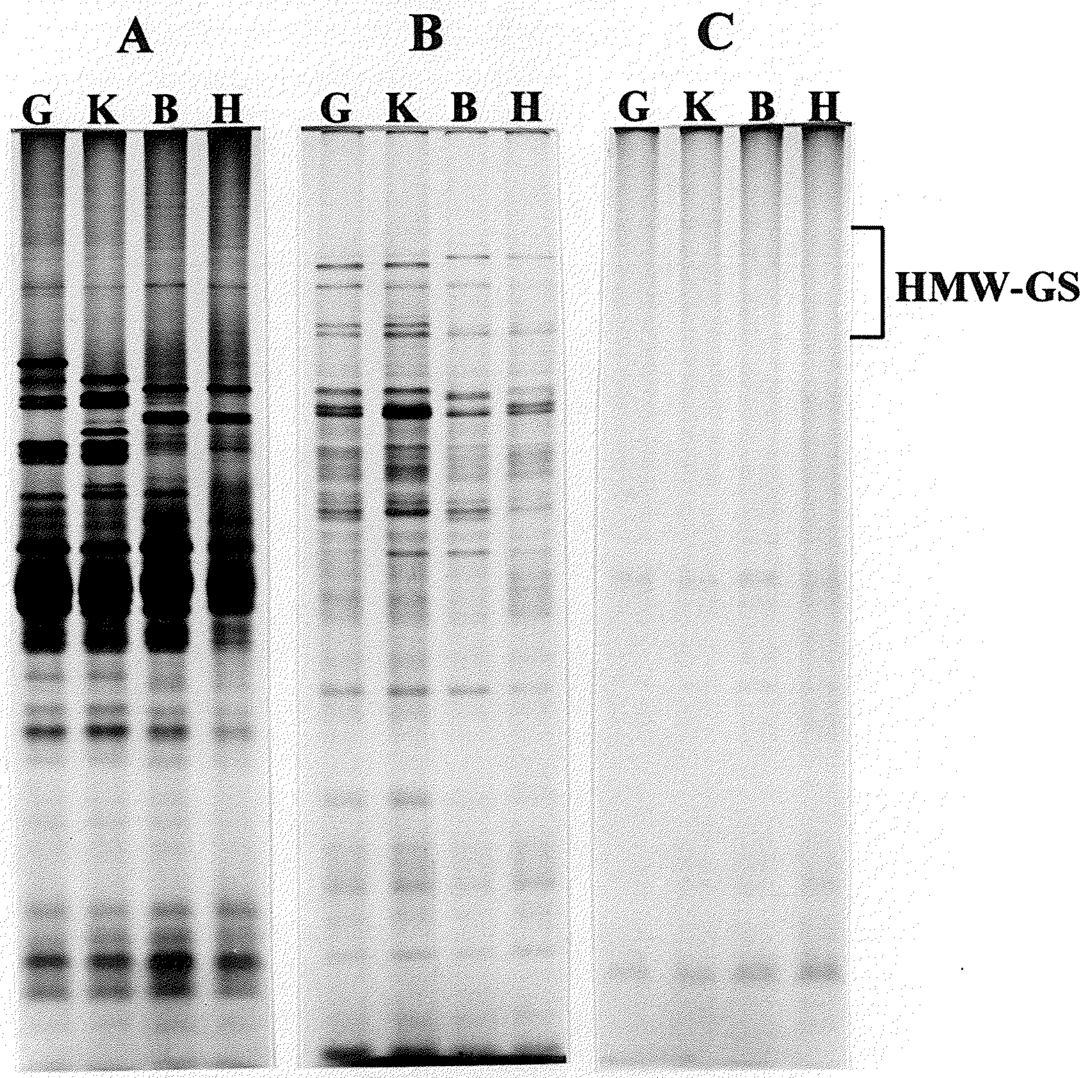

Glenlea
Katepwa
Biggar
Harus
TP PS PI FR
TP PS PI FR
TP PS PI FR
TP PS PI FR
2*
5
7
8
10
5
10
2*
5
7*
9
10
5
10
1
2
7
8
12
2
12
1
2
7*
9
12
2
12
HMW-GS

It was observed that very little protein was solubilized by the third extraction with 50% 1-propanol/1%DTT (results not shown), indicating that the final residue protein was insoluble in this solvent. One might surmise that in the absence of a strong denaturing agent such as SDS not all disulfide bonds in glutenin would be exposed to the propanol/DTT reductant solution, hence, a substantial amount of protein remained in the final residue. To examine whether or not the residue proteins existed as reduced subunits or were still in the form of polymers, the residue was treated with NEMI, to inactivate any remaining DTT, before extracting with SDS solution without reductant. First, we examined whether or not the NEMI can fully alkylate and inactivate DTT. The results showed that the mixture of DTT and NEMI in 50% 1-propanol had no reducing power as indicated by the absence of HMW-GS in the extract (Fig. 4.2 A). The SDS-PAGE patterns of the alkylated residue without further reduction (Fig. 4.2 B) were similar to those in Fig. 4.1 (lanes FR) which were fully reduced in the presence of SDS. This indicates that the final residue proteins revealed by SDS-PAGE exist as reduced subunits before the SDS extraction for analysis. The insolubility of final residue protein (in 50% 1-propanol/1% DTT) may be due to strong non-covalent interactions between specific subunits as indicated by the presence of noticeable protein staining in the gel slab slots which was evident in the reduced SDS-PAGE results of the residue (Fig. 4.1, lanes FR). That SDS was required to solubilize these proteins suggests that hydrophobic interactions may be responsible. The *Glu-D1* subunits appear to be quite important in maintaining such interactions, since even after three extractions with 50% 1-propanol under reducing conditions, these subunits still remained in the residue. The reason why a portion of the

Glu-D1 subunits were easily solubilized by 50% 1-propanol/1%DTT, while another portion remained in the final residue even after exhaustive extraction is not clear.

SDS-PAGE (Fig. 4.1, lanes FR) also revealed that the 50% 1-propanol/DTT insoluble residue contained quite a large proportion of non-prolamin proteins which is consistent with results of Gupta et al (1991b). When the 50% 1-propanol insoluble residue was extracted with SDS solution without reductant, electrophoregrams (Fig. 4.2 C) showed that this residue contained essentially no monomeric proteins. This result indicates that the remaining non-prolamin proteins in the final residue were originally associated with the polymeric protein fraction. Similar polypeptides have been previously observed in Osborne glutenin fractions (Bietz et al 1975), glutenin purified using dimethyl sulfoxide (Burnouf and Bietz 1989), and in the polymeric fraction of wheat flour protein (Gupta and Shepherd 1987, Gupta et al 1993). Genetic control and biochemical properties of these proteins were studied by Gupta et al (1991b). However, it is not known whether these non-prolamin polypeptides are covalently linked with glutenin subunits forming part of the native glutenin complex, or whether they represent a distinct polymer fraction that is highly insoluble.

Fig. 4.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing condition of flour proteins in various extracts. A, mixture of 1% dithiothreitol (DTT)/5% N-ethylmaleimide (NEMI)/50% 1-propanol soluble; B, SDS soluble of alkylated final residue; C, SDS soluble of 50% 1-propanol insoluble residue. G, Glenlea; K, Katepwa; B, Biggar; and H, Harus. HMW-GS, high molecular weight glutenin subunits.


A
B
C
G K B H
G K B H
G K B H
HMW-GS

Relationship Between Protein Solubility and Functional Properties

Table 4.3 shows the relationships between various flour quality parameters and the relative proportions of each protein fraction. The percentage of flour protein solubilized by 50% 1-propanol was very strongly negatively correlated with loaf volume and all measured physical dough properties except extensigraph extensibility. Conversely, the 50% 1-propanol/1%DTT soluble protein was very significantly positively correlated with dough strength and loaf volume. Similar correlations have also been reported between breadmaking quality parameters and the proportion of glutenin remaining unextractable (presumably on account of its molecular size), in solvents such as 3M urea (Pomeranz 1965), dilute acetic acid (Orth and Bushuk 1972) and SDS (Moonen et al 1983).

The final residue protein was negatively but not significantly related to quality parameters (Table 4.3). The negative, albeit weak, relationship between the final residue protein and quality is consistent with results obtained by MacRitchie (1987) for the more insoluble fractions of gluten. This fraction is a mixture of some glutenin subunits and non-prolamin components and originally from the 50% 1-propanol insoluble residue. Their contribution to the formation of larger-sized polymers is not clear.

TABLE 4.3

Linear Correlation Coefficients Between Percent Protein in Sequential Extracts and Quality Parameters for Seven Wheat Flours[a]

Quality Parameter	50% 1-propanol	50% 1-propanol /1%DTT	Residue
DDT	-0.95***	0.90**	-0.56
WIP	-0.98***	0.94**	-0.65
Rmax	-0.98***	0.94**	-0.64
Ext	-0.61	0.69	-0.79*
Area	-0.99***	0.97***	-0.74
LV[b]	-0.85*	0.83*	-0.60

[a]symbols *, **, and *** represent significant correlation at 5, 1, and 0.1% probability, respectively.
[b]loaf volume, other abbreviations used as in Table 4.1.

The 50% 1-propanol soluble fraction contained essentially all of the monomeric proteins and some glutenin. It was therefore unclear whether the strong negative relationship that was found between this fraction and breadmaking quality was due to either the monomeric protein or glutenin component or their combination. Accordingly, monomeric proteins and glutenin components of this fraction were isolated and quantified according to the procedure developed in Chapter 3. Table 4.4 shows that there was relatively little intercultivar variation in the concentration of monomeric proteins in the 50% 1-propanol soluble fraction. From 48-52% of flour protein constituted monomeric protein. The coefficient of variation (CV) was 2.9%. In contrast, the intercultivar variation in the proportion of 50% 1-propanol soluble glutenin was considerable (CV = 25.9%). Not surprisingly, therefore, correlation analysis showed that there was no significant relationship between the monomeric protein contents and quality parameters. In contrast, a very significant negative relationship existed between the amount of soluble glutenin and dough strength (Table 4.5).

It has been shown that the viscoelasticity of gluten can be greatly modified by changing the gliadin/glutenin ratio (Khathar et al 1995, Janssen et al 1996). However, this compositional factor is not always related to quality parameters since the size distribution of polymeric protein has a larger effect (Gupta et al 1993) and variation of the ratio of monomeric to polymeric protein is usually small between cultivars as found in this study. The strong relationship between soluble glutenin and quality is consistent with previous results that more glutenin was extracted by 50% 1-propanol in weak flours than strong ones (Kruger et al 1988, Marchylo et al 1989). The soluble glutenin is likely

much smaller in average molecular size than the insoluble glutenin. The latter fraction as represented by 50% 1-propanol insoluble polymeric protein (which can be further fractionated into two fractions after reduction as described above), significantly positively correlated with dough strength. It has been suggested that not all of the polymeric protein contributes to dough strength, *viz.* only a fraction above a certain molecular size, i.e. the critical molecular size for effective entanglements (MacRitchie 1992). The parallel influence of both soluble and insoluble glutenin in explaining so much of the variation in breadmaking quality attributes of the samples derives from the high interdependence of these two protein fractions (the correlation coefficient between the soluble and insoluble polymeric proteins was -0.95***).

Considering the data in Tables 4.2 and 4.4 together, it is interesting to note that the flour sample with the highest ratio of insoluble-to-soluble glutenin, as well as marginally the lowest concentration of monomeric and residue proteins was Glenlea which possesses very strong dough mixing characteristics.

TABLE 4.4

Percent of Monomeric Proteins, 50% 1-Propanol Soluble and Insoluble Glutenins in Seven Wheat Flours[a,b]

Flour	Monomeric Protein	Soluble Glutenin	Insoluble Glutenin
Glenlea	48.3 ± 0.7d	9.6 ± 0.5f	42.1 ± 0.6a
Roblin	51.5 ± 0.5ab	11.0 ± 0.4e	37.5 ± 0.4c
Katepwa	51.3 ± 1.1ab	12.6 ± 0.7d	36.1 ± 0.5d
Biggar	48.5 ± 0.7d	12.4 ± 0.5d	39.1 ± 0.6b
AC Karma	50.2 ± 0.4bc	15.5 ± 0.8c	34.3 ± 0.3e
SWS-52	49.8 ± 0.9c	19.4 ± 0.7a	30.8 ± 0.4f
Harus	51.9 ± 0.6a	17.8 ± 1.0b	30.3 ± 0.3f
Mean	50.2	14.0	35.7
CV[c]	2.9%	25.9%	12.0%

[a] Percent of the sum of the proteins in all fractions.

[b] Means of three replicates ± standard deviation, means with the same letter are not significantly different ($\alpha = 0.05$) between cultivars within a fraction.

[c] Coefficients of variation

TABLE 4.5

Linear Correlation Coefficients Between Percent of Monomeric Proteins, 50% 1-Propanol Soluble and Insoluble Glutenins and Quality Parameters for Seven Wheat Flours[a,b]

Quality Parameter	Monomeric Protein	Soluble Glutenin	Insoluble Glutenin
DDT	-0.55	-0.92**	0.96***
WIP	-0.53	-0.95***	0.98***
Rmax	-0.65	-0.91**	0.98***
Ext	-0.32	-0.59	0.60
Area	-0.64	-0.92**	0.99***
LV	-0.23	-0.89**	0.83*

[a]symbols *, **, and *** represent significant correlation at 5, 1, and 0.1% probability, respectively.
[b]abbreviations used as in Tables 4.1 and 4.3

Conclusions

Four distinct protein fractions were obtained from the fractionation procedures used in this study: one monomeric protein fraction, and three subfractions of polymeric protein which include 50% 1-propanol soluble glutenin (precipitated by 70% 1-propanol), 50% 1-propanol insoluble glutenin (soluble in 50% 1-propanol after reduction) and final residue protein. There was little variation in the amount of monomeric and residue protein fractions for the cultivars examined. The protein fractions with high intercultivar variability were soluble- and insoluble-glutenins. They were very closely related to dough properties and breadmaking quality. The strength of the statistical relationships between the insoluble glutenin concentration and quality, combined with the ease with which this glutenin fraction can be isolated from flour and quantified (in contrast to soluble glutenin) strongly suggest its utility as a test for discerning protein quality differences among different genotypes. The flour sample with the highest ratio of insoluble to soluble glutenin, as well as marginally the lowest concentration of monomeric and residue proteins, was Glenlea which possesses very strong dough mixing characteristics. These compositional results provide new information to explain the biochemical nature of the extra strong dough properties of Glenlea wheat. The observation that *Glu-D1* HMW subunits are dominant in the final residue compared with other HMW glutenin subunits indicates their uniqueness and draws more attention to the *Glu-D1* locus in relation to dough strength (Payne 1987).

Chapter 5

QUANTITATIVE AND QUALITATIVE SUBUNIT COMPOSITIONS OF 50% 1-PROPANOL SOLUBLE AND INSOLUBLE FRACTIONS OF WHEAT GLUTENIN

Abstract

This study was undertaken to identify factors that determine the solubility of wheat glutenin in 50% 1-propanol which has been widely used as a solvent of gluten proteins in studies of breadmaking quality. The glutenin components of flours of seven Canadian wheat cultivars of diverse dough strength were fractionated into 50% 1-propanol-soluble (50PS) and -insoluble (50PI) fractions. The 50PS glutenin was isolated from monomeric proteins in this fraction by precipitation with 70% 1-propanol, and further purified with 0.75 M sodium iodide to remove contaminating ω-gliadins. The 50PI glutenin was extracted as reduced subunits from the 50PI residue. Quantitative and qualitative subunit compositions of the two glutenin fractions were determined by reversed-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Results showed that there was essentially no qualitative difference in subunit composition of the two fractions regardless of the cultivar sample. Also, there was no difference in the relative amounts of the high molecular weight (HMW) and the low molecular weight (LMW) glutenin subunits (GS) determined by RP-HPLC. Results for all seven cultivars showed that compared to 50PS glutenin, 50PI glutenin contained a greater proportion of *Glu-1Dx* subunits, especially *Glu-1Dx5*, and a lower proportion of *Glu-1Bx* subunits. The proportions of *Glu-1Ax*

subunits (i.e. *Glu-1Ax1* and *Glu-1Ax2**), *Glu-1By* subunits (i.e. *Glu-1By8* and *Glu-1By9*), and *Glu-1Dy* (i.e. *Glu-1Dy10* and *Glu-1Dy12*) were relatively constant. For two of the seven cultivars (Glenlea and AC Karma), the proportion of a D-group LMW-GS (D*) in total LMW-GS in 50PS glutenin was more than two times their corresponding proportion in 50PI glutenin. The results obtained in this study indicate that *Glu-1Dx* subunits are crucial to the formation of less soluble glutenin (larger molecular size) which, quantitatively, appears to be directly related to dough strength.

Introduction

Wheat flour glutenin can be separated into two broad fractions based on their solubility in various solvents. Generally, the insoluble glutenin is presumed to be of larger molecular size than the soluble component (Gupta et al 1993). It is well documented that glutenins of wheat cultivars with diverse dough strength differ in solubility in dissociating solvents (for review see Weegels et al 1996). Accordingly, it is essential to know the differences in chemical and physical structures that determine solubility to develop a sound model for the molecular basis of breadmaking quality.

The structure/functionality of wheat flour glutenin has been studied using a variety of solvents. Beckwith et al (1966) first recognized and isolated an ethanol-soluble fraction of glutenin. This fraction was further characterized by workers at the USDA, Peoria, IL laboratory (Nielsen et al 1968; Bietz and Wall 1973, 1980; Huebner and Bietz, 1993). Based on electrophoretic, amino acid composition and N-terminal sequence results, they found that ethanol-soluble glutenin after reduction produced polypeptides identical to the LMW subunits of unfractionated glutenin. No HMW-GSs were reported in regard to the composition of ethanol-soluble glutenin. In contrast, subsequent research showed that alcohol-soluble glutenin also contained HMW subunits (Field et al 1983a; Kruger et al 1988; Chakraborty and Khan 1988a; Gupta and Shepherd 1990). Payne and Corfield (1979) showed that glutenin fractions of different molecular weight contained the same HMW and LMW subunits, but the proportions of the subunits changed. As the molecular size of the glutenin increased, the proportion of HMW-GS increased. Based on the results of extraction with 1.5% SDS solution and fractionation by precipitation/redissolution and

gel filtration techniques, Graveland et al (1985) reported that the two major glutenin fractions (glutenin I and II) had the same qualitative and quantitative subunit composition, and that the third minor fraction of lowest molecular size (glutenin III) did not contain HMW subunits. Gupta et al (1993) found that SDS-unextractable glutenin had a significantly higher ratio of HMW- to LMW-GS than the extractable fraction as determined by densitometric analysis of the stained SDS-PAGE gels. They postulated that molecular size increased as this ratio increased. Popineau et al (1994) prepared eleven gluten subfractions by sequential extraction of gluten in dilute HCl, and the subfractions were examined by RP-HPLC. They found that the total content of HMW-GS generally increased as gluten extractability decreased, but the proportions of individual HMW-GS in each fraction were essentially the same. Weegels et al (1995) found no difference in the proportions of the HMW and LMW subunits between 1.5% SDS unextractable glutenin and the Osborne glutenin fraction (70% ethanol insoluble) as determined by RP-HPLC. Based on results of RP-HPLC and densitometric analysis of SDS-PAGE, Dupuis et al (1996) found that the 0.05 N acetic acid soluble and insoluble fractions of glutenin were similar in subunit composition and content, with slightly less *Glu-1D* subunits (5+10) in the acetic acid soluble glutenin.

In light of the above, conclusions about the composition of glutenin reached by various workers are not in agreement. A possible source of variation in glutenin subunit composition is contamination by monomeric proteins which could be determined as LMW-GS by both SDS-PAGE and RP-HPLC analytical approaches. In the present study, wheat flour glutenin was first fractionated into 50PS and 50PI fractions. The 50PS

glutenin was then separated from other flour proteins in the 50PS fraction, and purified. The 50PI glutenin was extracted from the residue with 50% 1-propanol containing 1% (w/v) dithiothreitol (DTT) as reductant. Quantitative and qualitative subunit compositions of the two glutenin fractions were determined by RP-HPLC and SDS-PAGE. Seven Canadian wheat cultivars differing widely in dough strength were used to obtain information about the compositional differences between 50PS and 50PI glutenins in relation to breadmaking quality.

Materials and methods

Flour Samples

Flours of seven wheat cultivars of widely different baking strength were used in this study. Pertinent milling details and the technological properties of the flours are described in Chapter 4.

Chemicals and Solvents

All chemicals used were ACS grade or better. The following solutions were used for extraction of flour proteins for RP-HPLC or SDS-PAGE: "A", 50% 1-propanol (BDH, HPLC grade); "B", 0.08M Tris-HCl buffer, pH 7.5, containing 50% (v/v) 1-propanol; "C", solvent "B" containing 1% (w/v) DTT; and "D", solvent "B" containing 14% (v/v) 4-vinylpyridine (Sigma Chemical Co., ACS grade).

Extraction of Flour Proteins

Flour (100 mg) was mixed with 0.5 ml of 50% 1-propanol for 1 h at room temperature (23±1 °C) in a microcentrifuge tube with intermittent vortexing, and centrifuged for 3 min at 2,200 g. The supernatant was carefully transferred to another microcentrifuge tube with a pipette, and the residue was sequentially extracted with 0.3 ml and 0.2 ml of solvent "A" for 30 min per extraction, and centrifuged for 3 min at 2,200*g* and 15,000*g*, respectively. The residue remaining after these extractions was extracted with 0.2 ml of solvent "C" at 60 °C for 1 h. For RP-HPLC, the extraction with solvent "C" was followed by alkylation with 0.2 ml of solution "D" at 60 °C for 15 min prior to analysis. For SDS-PAGE, 0.1 ml of the supernatant obtained after extraction with solvent "C" was evaporated to dryness in a Speed Vac Concentrator and redissolved in 0.2 ml of buffer solution (pH 6.8), comprising 0.063 M Tris-HCl, 2% SDS, 1% DTT, 20% glycerol and 0.01% pyronin Y (SDS-PAGE buffer).

Isolation and Purification of Soluble Glutenin

A substantial amount of glutenin was extracted from flour together with essentially all monomeric proteins by solvent "A". This 50PS glutenin was precipitated by adding to a 0.5 ml aliquot of pooled supernatants, 0.34 ml 1-propanol to bring the final 1-propanol concentration to 70% (see Chapter 3). The mixture was vortexed and allowed to stand at room temperature for 1 h. The precipitated glutenin, obtained after centrifugation (15,000*g*, 10 min), was further purified by extraction (twice) with 1 ml of 0.75 M NaI (1 h and 30 min, respectively) to remove the co-precipitated monomeric

proteins (mainly ω-gliadins). The resulting residue was washed twice with distilled deionized water (DDW) for 2 min and centrifuged (15,000*g*); any remaining water was removed by careful aspiration using a pipette. For RP-HPLC, this purified glutenin was reduced with 0.1 ml of solvent "C" for 1 h at 60 °C followed by alkylation with 0.1 ml of solution "D" at 60 °C for 15 min. For SDS-PAGE, the glutenin was reduced by addition of 0.2 ml SDS-PAGE buffer (see below) which contained DTT.

SDS-PAGE

SDS-PAGE was carried out according to Ng and Bushuk (1987) except that the separating gel concentration was altered to 14% or 10%. The efficiency of the separation of the 50PS glutenin from the whole 50PS fraction was examined by SDS-PAGE under both reducing and non-reducing conditions.

RP-HPLC

For RP-HPLC of glutenin subunits, the separation conditions were as described in Chapter 3.

Collection of RP-HPLC Peaks for SDS-PAGE

The HMW-GS separated by RP-HPLC were identified after SDS-PAGE analysis according to the nomenclature of Payne and Lawrence (1983) and Marchylo et al (1992). To collect sufficient protein for SDS-PAGE, the multiple sample injection method of Marchylo and Kruger (1988) was used. Peaks in the HMW-GS region were collected and

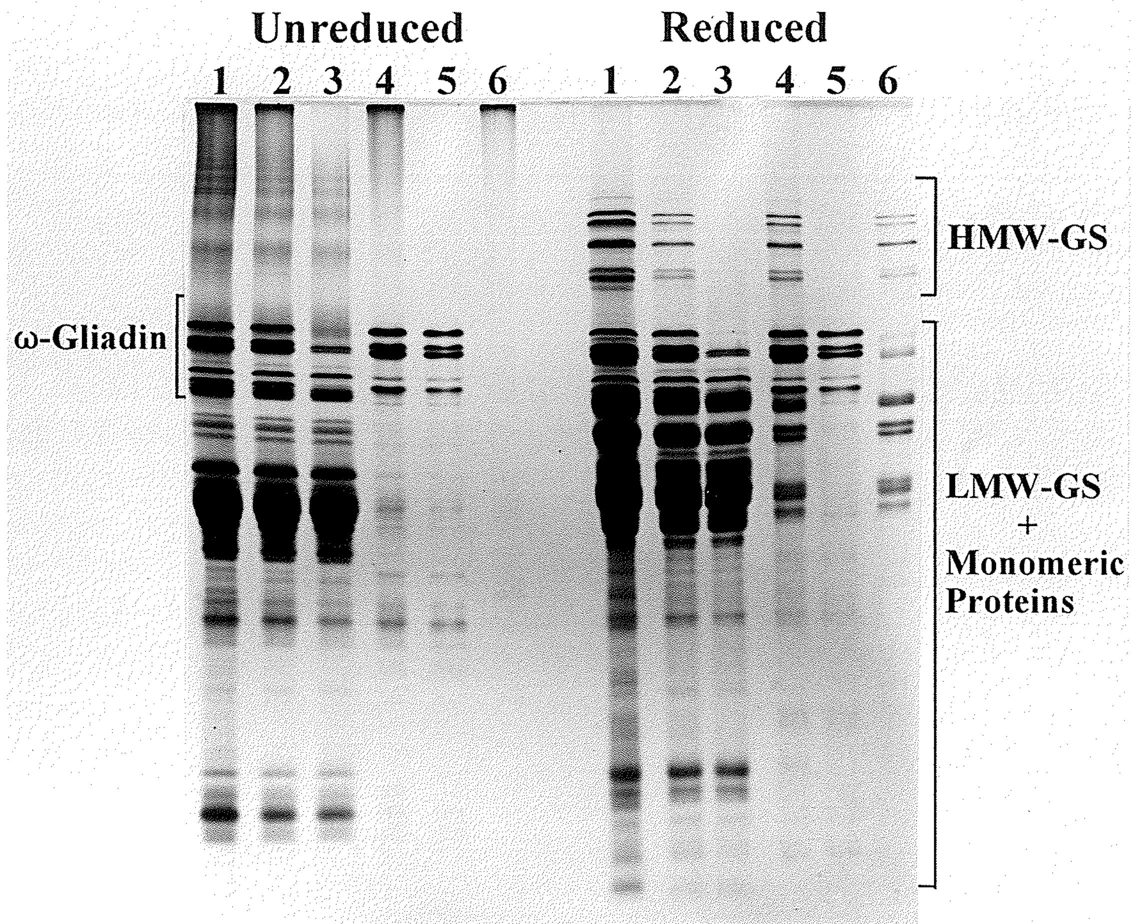
evaporated to dryness in a Speed Vac concentrator. Samples were redissolved in 100 μl of SDS-PAGE buffer and a 12 μl aliquot was analyzed by SDS-PAGE.

Results and Discussion

Isolation and Purification of 50PS Glutenin

Before analyzing glutenin subunits by RP-HPLC or SDS-PAGE, it was necessary to isolate and purify the glutenin extracted in 50PS fraction which contains a mixture of monomeric and polymeric proteins (see Chapter 3). To achieve this, the isolation procedure developed in Chapter 3 was used. The SDS-PAGE results for one wheat cultivar (Katepwa, Fig. 5.1) showed that the supernatant, after precipitation of 50PS glutenin with 70% 1-propanol without reduction, contained essentially no protein in the slot, and no HMW glutenin subunits, after reduction (Fig. 5.1, lane 3). This result indicated that all of the 50PS glutenin was precipitated by increasing the concentration of 1-propanol to 70%. However, the precipitated glutenin was still contaminated with non-glutenin proteins, mainly ω-gliadins (Fig. 5.1, lane 4). These monomeric proteins were selectively removed by extraction with 0.75 M NaI without losing any polymeric glutenin; as no glutenin was found in the supernatant of the 0.75 M NaI extraction and the residue contained no monomeric proteins (Fig. 5.1, lanes 5 and 6). Experiments with different concentrations (0.25 M - 1.0 M) of NaI showed that 0.75 M was the optimum concentration to remove the contaminating ω-gliadins (results not shown). Thus, the 50PS glutenin was completely recovered without contamination of any monomeric proteins.

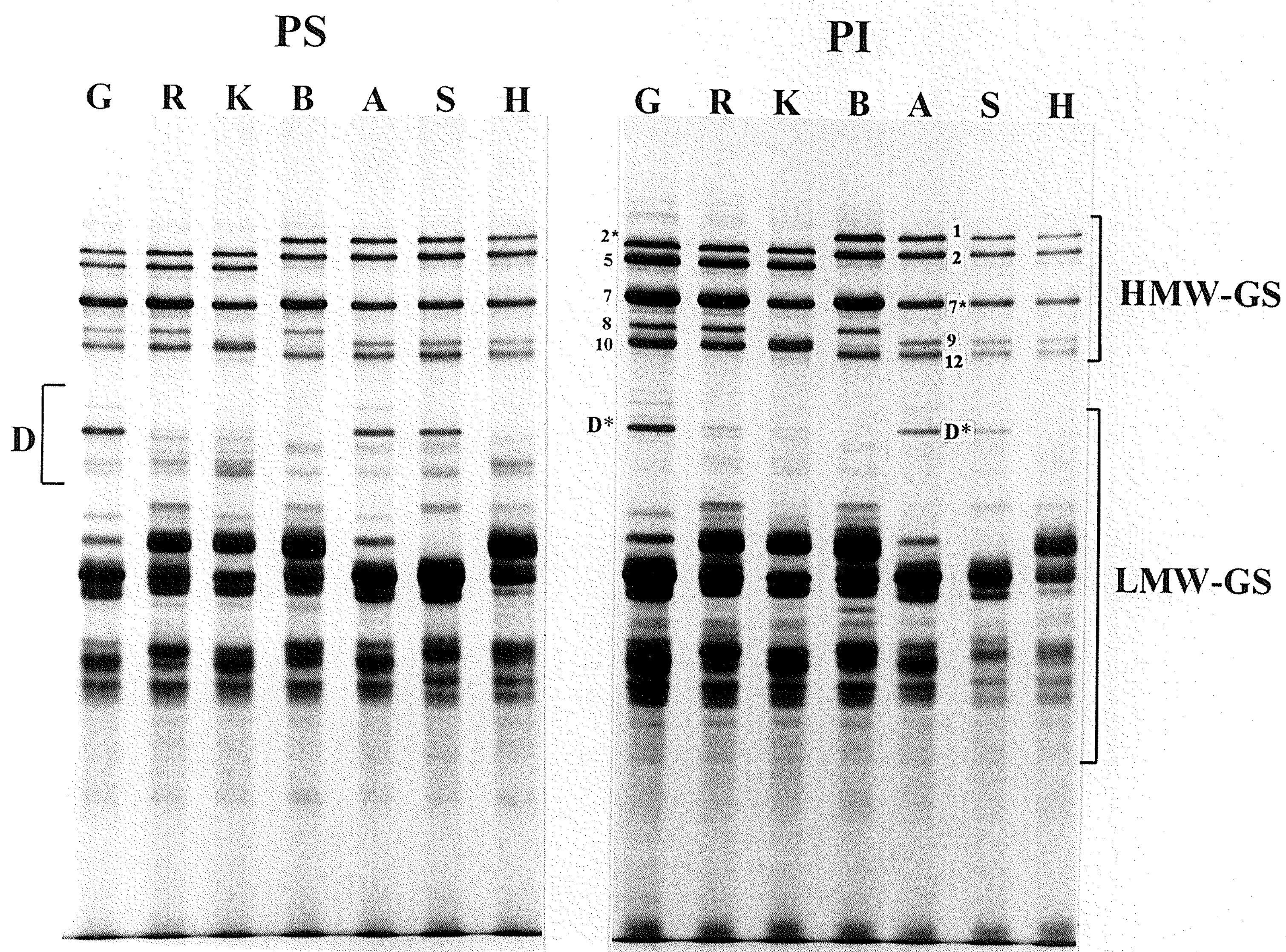
Fig. 5.1. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis under non-reducing and reducing conditions of various fractions of Katepwa flour. 1, 2% SDS soluble (control); 2, 50% 1-propanol soluble; 3 and 4, 70% 1-propanol supernatant and precipitate of 50% 1-propanol soluble, respectively; 5 and 6, 0.75 M NaI soluble and insoluble of 70% 1-propanol precipitate, respectively. HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits.


Unreduced
Reduced
1 2 3 4 5 6
1 2 3 4 5 6
ω-Gliadin
HMW-GS
LMW-GS
+
Monomeric
Proteins

SDS-PAGE of 50PS and 50PI Glutenin

The HMW-GS composition (Fig. 5.2) of the cultivar samples were as follows: Glenlea and Roblin, 2*, 7+8, 5+10; Katepwa, 2*, 7*+9, 5+10; Biggar, 1, 7+8, 2+12; AC-Karma, SWS-52 and Harus, 1, 7*+9, 2+12. The pattern of Katepwa glutenin did not show a clear resolution of subunits 9 and 10 in the 10% separating gel system which is optimized for resolution of LMW-GS. The relative amount of HMW-GS 7 (expressed as per cent of total HMW subunits) was significantly higher than that for subunit 7* (Marchylo et al 1992). 50PS and 50PI fractions of glutenin (Fig. 5.2) had essentially the same composition of both HMW- and LMW-GS, except that the 50PS fraction had quantitatively more minor components in the D zone of SDS-PAGE (Jackson et al 1983) compared to that of 50PI fraction. Accordingly, there was little qualitative difference in subunit composition between the two glutenin fractions of the seven cultivars examined. Possible quantitative differences in the relative proportions of individual subunits or groups of subunits between the two fractions were further examined by RP-HPLC.

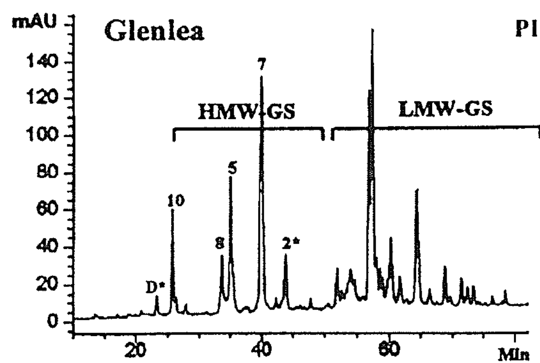
Fig. 5.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of reduced 50% 1-propanol soluble (PS) and insoluble (PI) glutenin fractions of seven cultivars. G, Glenlea; R, Roblin; K, Katepwa; B, Biggar; A, AC Karma; S, SWS-52; and H, Harus. HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits. D, D group LMW-GSs; D*, a ω-gliadin type D subunit of glutenin present in Glenlea and AC Karma. The HMW-GSs of Glenlea (G) and AC Karma (A) are numbered according to the nomenclature of Payne and Lawrence (1983) and Marchylo et al (1992).

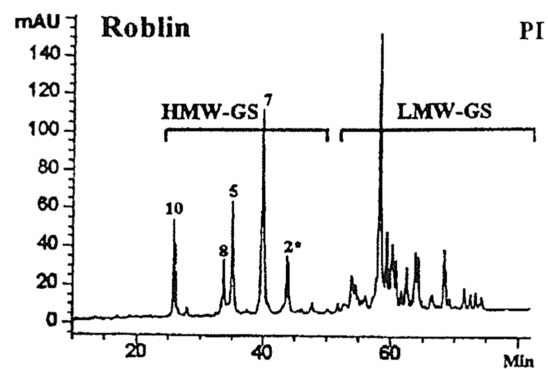

PS
PI
G R K B A S H
G R K B A S H
D
2*
5
7
8
10
D*
1
2
7*
9
12
D*
HMW-GS
LMW-GS

Quantitative and Qualitative Comparison of Subunits of 50PS and 50PI Glutenins by RP-HPLC

The RP-HPLC chromatograms of alkylated subunits of 50PS and 50PI glutenins of the seven cultivars showed that both HMW- and LMW-GS were effectively eluted in the 23-44% acetonitrile gradient used (Fig. 5.3). The first group of peaks, with elution times of 24-46 min, were the HMW-GS, and the second group comprising the LMW-GS had elution times greater than 46 min (see Chapter 3). The RP-HPLC chromatograms confirmed the electrophoretic results that the 50PS and 50PI glutenins had essentially the same qualitative subunit composition.

Fig. 5.3. Reversed-phase high-performance liquid chromatograms of reduced and alkylated subunits of 50% 1-propanol soluble (PS) and insoluble (PI) glutenin fractions of seven wheat cultivars. High molecular weight glutenin subunits of each cultivar are numbered based on electrophoretic results of Fig. 5.4. D*, a ω-gliadin type D subunit of glutenin present in Glenlea and AC Karma.


Glenlea
PI
mAU
HMW-GS
LMW-GS
10
8
5
7
2*
D*
Min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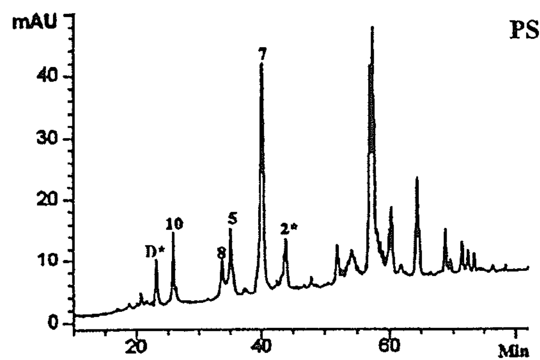
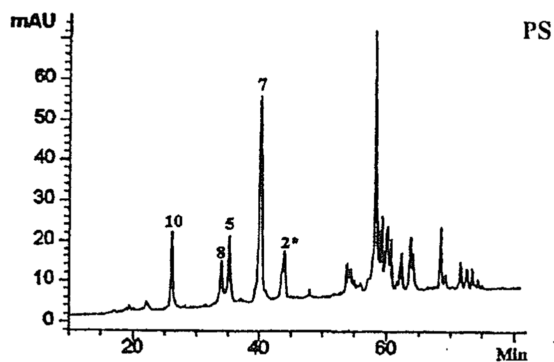
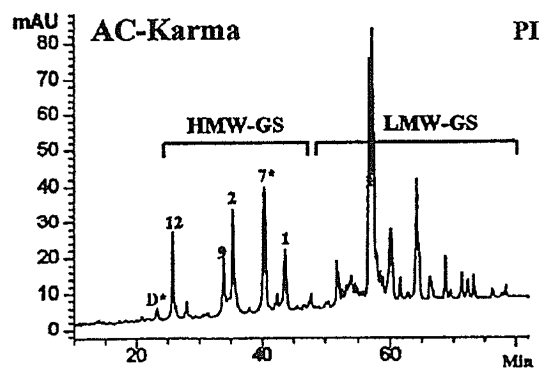
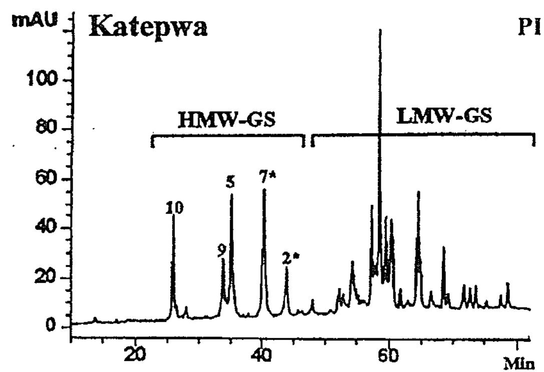
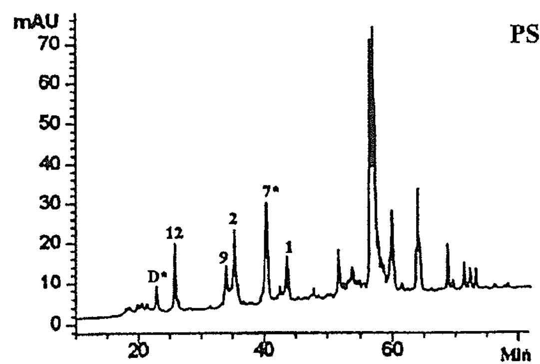
mAU
HMW-GS
LMW-GS
10
8
5
7
2*
Min

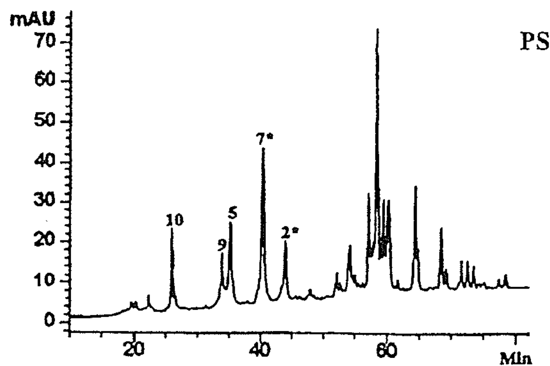

PS
mAU
D*
10
8
5
7
2*
Min

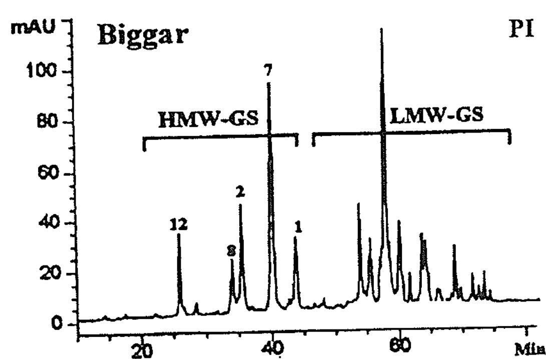

PS
mAU
10
8
5
7
2*
Min

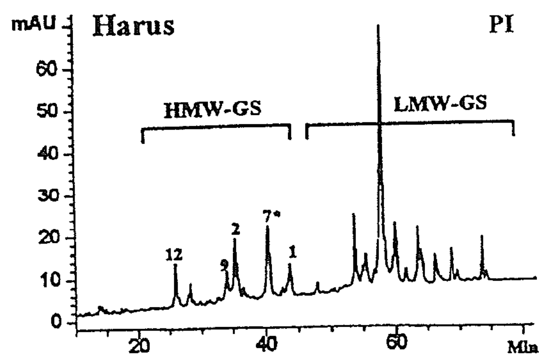

AC-Karma
PI
mAU
HMW-GS
LMW-GS
D*
12
9
2
7*
1
Min

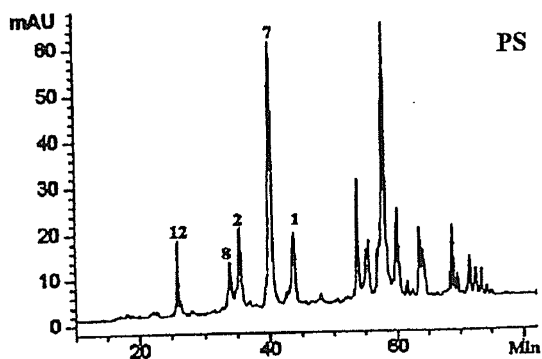

Katepwa
PI
mAU
HMW-GS
LMW-GS
10
9
5
7*
2*
Min

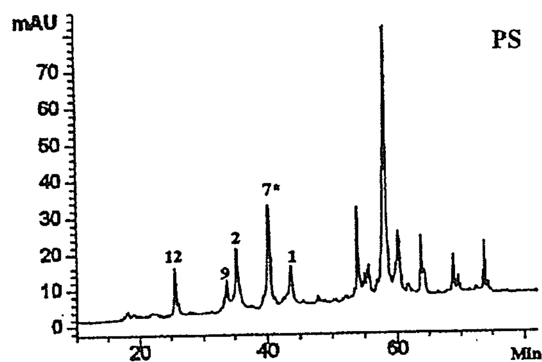

PS
mAU
D*
12
9
2
7*
1
Min

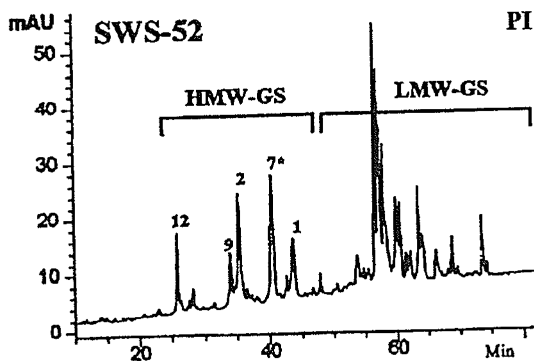

PS
mAU
10
9
5
7*
2*
Min

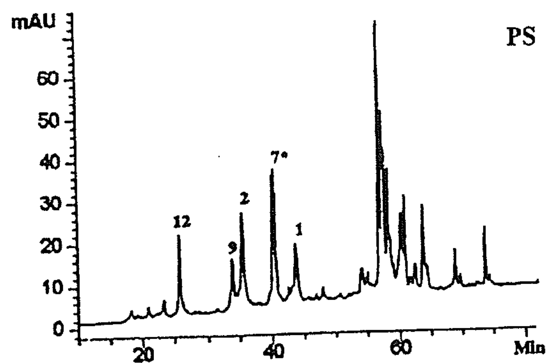

mAU
Biggar
PI
100
80
60
40
20
0
HMW-GS
LMW-GS
12
8
2
7
1
20
40
60
Min


mAU
Harus
PI
60
50
40
30
20
10
0
HMW-GS
LMW-GS
12
9
2
7*
1
20
40
60
Min


mAU
PS
60
50
40
30
20
10
0
12
8
2
7
1
20
40
60
Min

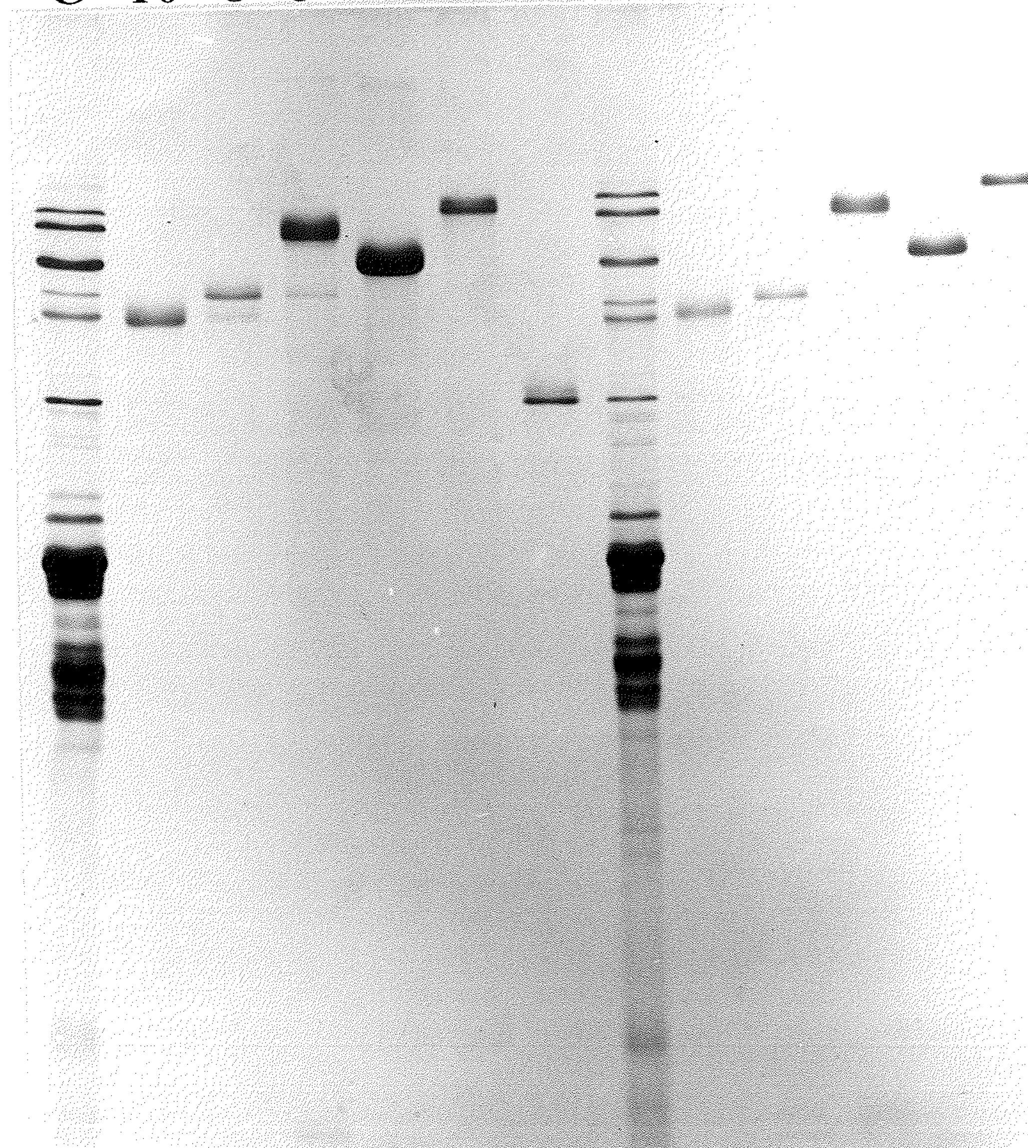

mAU
PS
70
60
50
40
30
20
10
0
12
9
2
7*
1
20
40
60
Min


mAU
SWS-52
PI
50
40
30
20
10
0
HMW-GS
LMW-GS
12
9
2
7*
1
20
40
60
Min


mAU
PS
60
50
40
30
20
10
0
12
9
2
7*
1
20
40
60
Min

Each peak of HMW-GS was collected and analyzed by SDS-PAGE to confirm its identity. Figure 5.4 shows typical results for cultivars Glenlea and AC Karma which together account for all of the HMW-GS present in the seven cultivars examined. The order of elution of the HMW subunits is the same as reported by Marchylo et al (1989) and Kawka et al (1992). The small peak which eluted prior to the HMW-GS in both Glenlea and AC Karma (Fig. 5.3) was identified here, for the first time, as a D group LMW-GS which, on SDS-PAGE, appeared as a single band migrating between the HMW- and LMW-GS. This component is labelled as D* on SDS-PAGE of 50PI glutenin (Fig. 5.2). By SDS-PAGE analysis, SWS-52, which contains a similar (albeit fainter) band with identical mobility to D*, did not clearly show an analogous peak eluting before the HMW-GS (Fig. 5.3). No further peak identification was done for this D-glutenin subunit of SWS-52 and other minor D-zone components present in the seven cultivars. D-type glutenin subunits are the slowest-moving group of the LMW-GS in SDS-PAGE and have the most acidic isoelectric points with pI of about 5 (Jackson et al 1983). They are coded by genes on the short arm of chromosome 1B or 1D (Jackson et al 1983). Masci et al (1993) reported that the D-group subunits have ω-gliadin type N-terminal amino acid sequences but are included in the glutenin fraction because they have at least one cysteine in their primary sequences. These workers speculate that D-subunits of glutenin may act as polypeptide chain terminators in the polymerization of subunits to form glutenin polymers. How this possibility would affect polymer size remains to be determined.

Fig. 5.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the high molecular weight glutenin subunits and a ω-gliadin type D group low molecular weight glutenin subunit (D*) of Glenlea and AC-Karma in peaks resolved by reversed-phase high-performance liquid chromatography in Fig. 5.3. Lane numbers are the subunit numbers according to Payne and Lawence (1983) and Marchylo et al (1992) and correspond to peak numbers in Fig. 5.3. C = reduced 50% 1-propanol insoluble glutenin (control).


Glenlea
AC-Karma
C 10 8 5 7 2* D* C 12 9 2 7* 1 D*

The contents of the HMW- and LMW-GS in the 50PS and 50PI glutenin fractions were determined by measuring the sum of peaks of individual HMW-GS and the total peak area of the LMW-GS region, respectively. The ratio of HMW- to LMW-GS of the two glutenin fractions (Table 5.1) were comparable for all seven cultivars examined. Glenlea and AC Karma, which appear to have identical LMW-GS composition, showed slightly higher ratios of HMW- to LMW-GS for the 50PI glutenin. Conversely, the 50PI glutenin in the two soft wheat cultivars (SWS-52 and Harus) contained slightly lower ratios of HMW to LMW subunits than their 50PS fraction. For the remaining three cultivars, the two glutenin fractions had essentially identical ratios of HMW- to LMW-GS. Unlike the results of previous studies (Payne and Corfield 1979, Gupta et al 1993), the direct relationship between solubility (molecular size) of glutenin and the ratio of HMW to LMW subunits was not found.

The relative proportions of individual HMW-GS in the total composition of HMW-GS of 50PS and 50PI glutenin fractions were also determined (Table 5.2). For the seven wheat cultivars examined, subunit *Glu-1Bx* (7 or 7*) was present in the greatest quantity, followed by subunit *Glu-1Dx* (2 or 5), the proportion of subunit *Glu-1By* (8 or 9) was the lowest. Results for the two glutenin fractions showed that 50PI glutenin contained a higher proportion of *Glu-1Dx* subunits (especially 1Dx5) and a lower proportion of *Glu-1Bx* subunit. In the case of Glenlea, e.g., the proportion of subunit 5 was 15.1% in 50PS glutenin and 22.9% in 50PI glutenin, while the proportion of subunit 7 was 51.1% in 50PS glutenin and 42.6% in 50PI glutenin. *Glu-D1* subunits, have been shown to have a distinct relationship to breadmaking quality (Payne 1987). The observed

TABLE 5.1

Ratios of High Molecular Weight to Low Molecular Weight Glutenin Subunits for 50% 1-Propanol Soluble (50PS) and Insoluble (50PI) Glutenin Fractions Obtained by Reversed-Phase High-Performance Liquid Chromatography[a]

Cultivar	50PS	50PI
Glenlea	0.49 ± 0.02a	0.54 ± 0.01b
Roblin	0.64 ± 0.02a	0.62 ± 0.01a
Katepwa	0.47 ± 0.01a	0.46 ± 0.01a
Biggar	0.54 ± 0.01a	0.53 ± 0.01a
AC-Karma	0.37 ± 0.01a	0.41 ± 0.01b
SWS-52	0.43 ± 0.01a	0.39 ± 0.01b
Harus	0.40 ± 0.02a	0.35 ± 0.02a

[a] Mean of triplicates ± standard deviation. Values with the same letter (between fractions within a cultivar) are not significantly different (α=0.05).

TABLE 5.2

Percentage (%) of Individual High Molecular Weight Glutenin Subunits (HMW-GS) in Total HMW-GS in the 50% 1-Propanol Soluble (50PS) and Insoluble (50PI) Glutenin Fractions[a]

Cultivar and HMW-GS	1Ax(1 or 2*)		1Bx(7 or 7*)		1By(8 or 9)		1Dx(2 or 5)		1Dy(10 or 12)	
	50PS	50PI	50PS	50PI	50PS	50PI	50PS	50PI	50PS	50PI
Glenlea (2*,7+8,5+10)	12.6±0.3a	10.6±0.2b	51.1±1.3a	42.6±0.1b	8.9±0.3a	9.7±0.2b	15.1±0.4a	22.9±0.4b	12.3±0.7a	14.1±0.4b
Roblin (2*,7+8,5+10)	15.9±0.6a	13.2±0.2b	50.0±0.5a	42.2±0.6b	9.7±0.5a	10.7±0.6a	14.1±0.3a	20.0±0.1b	10.4±0.7a	13.9±0.3b
Katepwa (2*,7*+9,5+10)	15.8±0.2a	12.3±0.2b	37.3±0.3a	30.9±0.4b	13.0±0.1a	13.8±0.4a	19.0±0.6a	25.6±0.6b	15.0±0.3a	17.4±0.3b
Biggar (1,7+8,2+12)	16.6±0.7a	15.1±0.2a	49.0±1.2a	44.0±0.2b	8.7±0.3a	8.9±0.1a	16.2±0.2a	20.7±0.1b	9.7±0.7a	11.3±0.3b
AC-Karma (1,7*+9,2+12)	15.1±0.9a	14.6±0.6a	33.4±1.1a	30.4±0.7b	12.0±0.3a	11.9±0.3a	25.1±1.3a	27.2±0.8a	14.4±1.1a	15.9±0.7a
SWS-52 (1,7*+9,2+12)	18.0±1.0a	16.8±0.4a	32.6±0.8a	29.8±0.2b	11.2±0.6a	10.7±0.1a	24.2±1.2a	28.1±0.6b	14.0±0.8a	14.6±0.0a
Harus (1,7*+9,2+12)	16.6±0.3a	16.0±0.3a	35.3±0.9a	29.5±0.3b	11.8±0.0a	12.2±0.8a	24.2±0.6a	27.9±0.7b	12.2±0.5a	14.4±0.4b

[a] Mean of triplicates ± standard deviation. Values with the same letter (between fractions within a cultivar) are not significantly different ($\alpha=0.05$).

higher proportions of *Glu-1Dx* subunits in 50PI glutenin suggests that these subunits may be important in the formation of the more insoluble glutenin (presumably larger polymers), the quantity of which has been shown to be positively correlated with breadmaking quality in many studies (for review see Weegels et al, 1996). Lawrence and Payne (1983) suggested that the *Glu-D1* HMW subunits may be involved in oligomer formation which precedes the assembly of the larger glutenin polymers.

It is of note that the magnitude of the difference in the proportion of HMW-GS 5 (Glenlea, Roblin and Katepwa) between the 50PS and 50PI glutenins was significantly larger than that of its allelic subunit 2 (Biggar, AC Karma, SWS-52 and Harus). The extra cysteine residue in *Glu-1Dx5* (Greene et al 1988) may have an effect of enabling larger glutenin molecules (more insoluble) to be formed. This hypothesis is consistent with the results of Marchylo et al (1989), who showed that extractabilities of other HMW-GS (in the form of polymeric glutenin) increased significantly in 50% 1-propanol when *Glu-D1* subunits (5+10) were genetically deleted. Gupta et al (1995) also showed that the contribution of the *Glu-D1* locus to the unextractable polymers was significantly greater than that of either *Glu-B1* or the *Glu-A1* locus. The relative proportions of the *Glu-1Ax* (1 or 2*), *Glu-1By* (8 or 9), and *Glu-1Dy* (10 or 12) subunits of the two glutenin fractions of the seven cultivars examined were relatively constant (Table 5.2), and hence they likely do not contribute to the well-known intercultivar variability in glutenin solubility.

As indicated earlier, cultivar Glenlea, which has the strongest dough mixing properties, has a very prominent band (D*) in the D zone of the SDS-PAGE pattern of

the LMW-GS (Fig. 5.2). Also, AC Karma contained this band but in much smaller quantity. This relatively unique D* subunit was quantified (by RP-HPLC) for Glenlea and AC Karma to examine its possible relationship to dough strength. Of the total area under the RP-HPLC profile for the LMW-GS, the D* subunit represented $3.7\pm0.4\%$ and $1.6\pm0.1\%$ in the 50PS and 50PI glutenin fractions of Glenlea, respectively, and $2.4\pm0.1\%$ and $1.0\pm0.2\%$ in the two fractions of AC Karma. The experimental error indicated is based on three determinations. The genetic and/or technological significance of this observation, if any, remains to be determined.

Conclusions

The correlation between glutenin solubility and breadmaking quality, first reported by Orth and Bushuk (1972), has been supported by many studies reported over the last two decades. Identification of the biochemical properties of glutenin, which determine its solubility or molecular size distribution, would be beneficial to devise strategies for breeding wheat varieties with improved breadmaking quality. In this study, wheat flour glutenin was first fractionated into 50PS and 50PI fractions. Quantitative and qualitative subunit compositions of the two glutenin fractions were determined by RP-HPLC and SDS-PAGE for seven cultivars of diverse breadmaking quality. The ratio of HMW- to LMW-GS of the two glutenin fractions was essentially constant for the seven cultivars. Results of the analysis for all cultivars showed that PI glutenin contained a greater proportion of *Glu-1Dx* subunits, especially *Glu-1Dx5*, and a lesser proportion of *Glu-1Bx* subunits than PS glutenin. The proportions of *Glu-1Ax* subunits (i.e. *Glu-1Ax1* and *Glu-*

*1Ax2**), *Glu-1By* subunits (i.e. *Glu-1By8* and *Glu-1By9*), and *Glu-1Dy* subunits (i.e. *Glu-1Dy10* and *Glu-1Dy12*) were essentially constant. The results obtained in this study suggest that *Glu-1Dx* subunits are crucial to the formation of more insoluble glutenin (higher molecular weight) which, quantitatively, appears to be directly related to dough strength.

Chapter 6

EFFECT ON DOUGH MIXING PROPERTIES OF ENRICHMENT OF BASE FLOURS WITH GLUTENIN AND GLIADIN FRACTIONS OF WHEAT CULTIVARS OF DIVERSE STRENGTH

Abstract

Gliadin- and glutenin-rich protein fractions were isolated from 0.5 M NaCl insoluble residues of three Canadian wheat flours by repeated extraction with distilled deionized water (DDW). The solvent (DDW) and physical treatment (mild magnetic stirring) used for preparation of the two fractions were very mild compared to methods based on dissociating solvents and high-shear mixing. Electrophoretic and compositional analysis showed that the separation of gliadin and glutenin was very effective. The gliadin- and glutenin-rich fractions caused significant weakening and strengthening, respectively, of base flour doughs. Gliadin-rich fractions isolated from three wheat cultivars with widely different dough mixing properties gave similar dough-weakening effects when they were added at equivalent protein levels to a very strong base flour (Glenlea). This result indicated that qualitative variation in gliadin composition did not contribute to intercultivar variations in dough mixing properties. In contrast, the degree of dough-strengthening effects of glutenin-rich fractions was cultivar dependent. The glutenin-rich fraction isolated from flour of the Canada Extra Strong Red Spring wheat Glenlea produced a significantly higher dough-strengthening effect than that of Katepwa, a Canada Western Red Spring wheat. The glutenin from Soft White Spring wheat SWS-

52 with weak dough properties produced the lowest dough-strengthening effect of the three cultivars examined. Results indicated that qualitative differences (presumably molecualr size) in glutenin is the principal factor in determining differences in dough mixing properties among wheat cultivars.

Introduction

There is considerable published evidence that intercultivar variation in flour strength (for breadmaking) is controlled mainly by differences in the glutenin protein of the flour (for review see Weegels et al 1996). One aspect of glutenin structure that appears to be important to breadmaking quality is the composition of the high molecular weight glutenin subunits (HMW-GS) (Payne 1987). The functional role of low molecular weight (LMW)-GS has received little attention until recently. Variation in LMW-GS may be related to variation in dough properties, but to a lesser degree than that of HMW-GS (Gupta et al 1995). It follows that the type, amount, and the relative proportion of subunits will determine the ultimate structure of glutenin as it exists in flour, and its functionality for breadmaking. While considerable structural information on glutenin has been accumulated (Kasarda 1989, MacRitchie 1992, Schofield 1994, Bushuk 1994), a coherent and widely accepted hypothesis concerning the relationship between structure and functionality has not yet been established.

It is well recognized that the dough mixing behaviour of flour involves both glutenin and gliadin proteins. The relative proportion of gliadin in bread wheat cultivars of diverse breadmaking potential is relatively constant (Orth and Bushuk 1972). Although several publications have reported the existence of significant associations between the presence of specific gliadin components and dough strength, there is no generally accepted relationship (for review see MacRitchie et al 1990). The qualitative variation in gliadin composition among different genotypes, albeit very extensive and useful in cultivar identification, does not appear to contribute significantly to intercultivar

differences in mixing and baking properties.

Most research on the role of specific type(s) of wheat polypeptides in relation to functional properties has been based on correlation studies, in which the proportion of wheat protein fractions are determined or allelic variations in protein composition are related to end use quality. However, even high correlations when found cannot establish cause and effect relationships. Fractionation and reconstitution studies have the potential to provide more direct evidence on the functionality of specific protein components. This study reports additional new information on the functionality of gliadin and glutenin proteins using a fractionation/reconstitution strategy that is substantially different from that used by other workers (Hoseney et al 1969a, 1969b; Goforth et al 1977; MacRitchie 1978, 1985, 1987; Booth and Melvin 1979; Preston and Tipples 1980). Relatively sharply defined gliadin and glutenin fractions were prepared by a "gentle" procedure which avoids the use of denaturing solvents such as alcohol or acid and rigorous physical shearing during extraction. Secondly, the natural range of wheat flour strength has been expanded by including the extra strong Canadian cultivar Glenlea. Thirdly, dough mixing properties were examined using a direct drive computerized mixograph which imparts sufficient mixing energy to fully develop the dough from flours of the strongest available bread wheat flours.

Materials and Methods

Wheat Flour Samples

Flours of three cultivars (Glenlea, Katepwa and SWS-52) differed widely in mixing and baking properties were used. The technological properties of the three flours have been described (Table 4.1, Chapter 4).

Preparation of Gliadin- and Glutenin-rich Flour Protein Fractions

Flour (20 g) was extracted with 0.5 M NaCl (200 ml) for 1 h at room temperature (23 ± 1°C), then centrifuged at 500*g* for 5 min. To remove residual salt, the residue was washed twice with 200 ml of distilled and deionized water (DDW) for 5 min each. This was followed by four sequential extractions with 100 ml of DDW for 1 h at room temperature with mild magnetic stirring. The four supernatants obtained by centrifugation (10,000*g*, 10 min) after each extraction were combined and freeze-dried. This material is referred to as the gliadin-rich (Gli-R) fraction. The water insoluble residue was carefully washed manually in a wash chamber of the Glutomatic system (Falling Number AB, Sweden) under a stream of distilled water to remove the starch. Washing was deemed complete when the washing water ran clear. The remaining residue was freeze-dried and is referred to as the glutenin-rich (Glu-R) fraction. The protein contents of Gli-R and Glu-R fractions were determined by the micro-Kjeldahl method (AACC 1983).

Electrophoresis

Gli-R and Glu-R protein fractions were analyzed by acid-polyacrylamide gel

electrophoresis (A-PAGE) according to Sapirstein and Bushuk (1985), and sodium dodecyl sulfate (SDS)-PAGE according to Ng and Bushuk (1987) with the separating gel concentration of 14%. For comparison, 0.5 M NaCl insoluble proteins prepared from each flour were also analyzed.

Quantitation of Gliadin and Glutenin in Isolated Fractions

Freeze-dried Gli-R fraction (10 mg) was dissolved in 0.5 ml 50% 1-propanol. Glutenin in this fraction was precipitated by adding 0.34 ml of 1-propanol to bring the final 1-propanol concentration to 70% (v/v) (see Chapter 3). After centrifugation (15,000*g*, 10 min), the precipitate was extracted twice (1 h and 30 min, respectively) with 0.75 M NaI (1 ml) to remove the coprecipitated ω-gliadins (see Chapter 5). The concentration of glutenin in the Gli-R fraction was calculated by subtracting the amount of protein in the 70% 1-propanol supernatant and 0.75 M NaI soluble, which were combined and quantified by micro-Kjeldahl analysis (AACC 1983).

The freeze-dried Glu-R fraction (20 mg) was extracted twice with 0.75 M NaI (1 ml) in a microcentrifuge tube at room temperature (see Chapter 5). The protein contents of the pooled supernatants (gliadin) and the residue (glutenin) were determined by the micro-Kjeldahl method.

Dough Mixing Properties

The functionality of Gli-R and Glu-R fractions in dough mixing were separately evaluated by adding them to two different base flours. The mixing properties of the

fortified base flours were measured with a computerized 2-g mixograph (National Manufacturing Division, TMCO, Lincoln, NE). Base flours of Glenlea and SWS-52 were chosen for the additions of Gli-R and Glu-R fractions, respectively. This enabled the anticipated dough-weakening and dough-strengthening responses of gliadin and glutenin to be evaluated. Protein fractions were added to base flours to increase the protein level by 0.5, 1.0, 1.5, 2.0 and 2.5 percentage units. The mixing properties of Glenlea base flour (2.0 g, 14% m.b.) and Gli-R fortified Glenlea flours (2.0 g including the weight of protein fractions added) were all determined at 65% water absorption level. For the SWS-52 base flour and Glu-R fortified SWS-52 flours, the mixing properties were determined at 60% water absorption. Mixing time to peak dough development (MDT) and work input to peak (WIP) were derived automatically from the midline by using the accompanying Mixsmart software. All mixing tests were performed in triplicates. The mean values were reported.

Results

Protein Contents and Composition of Gli-R and Glu-R Fractions

The protein contents (Table 6.1, as is basis) of freeze-dried Gli-R and Glu-R fractions were very similar for all three cultivar samples, and averaged 89% and 72%, respectively.

SDS-PAGE was run under reducing and non-reducing conditions to examine the protein composition of the isolated fractions (Fig. 6.1). The presence of only trace amounts of HMW subunits of glutenin in the reduced Gli-R fraction (Fig. 6.1, lanes 2),

confirmed that this fraction contained very little glutenin. In contrast, the Glu-R fraction appeared to contain a higher level of contaminating protein, i.e. some ω-gliadin components and trace amounts of α-, β-, and γ-gliadins as revealed by SDS-PAGE under non-reducing conditions (Fig. 6.1, lanes 3). This observation is in close agreement with data shown in Table 6.1 which indicates that gliadins in the Glu-R fraction were present at about twice the level (~10%) than that of glutenin protein in the Gli-R fraction (~5%). The strong staining of the HMW-GS in the SDS-PAGE of Glu-R fraction under reducing conditions is also in agreement with data shown in Table 6.1; this fraction consisted of mainly glutenin (~90%). Further comparison of the monomeric protein composition of these fractions by APAGE analysis (Fig. 6.2) confirmed that the Gli-R fraction contained mostly gliadins and Glu-R fraction contained some ω-gliadin components and trace amounts of α-, β- and γ-gliadins. These results indicated that a very good separation of gliadin and glutenin protein was obtained by repeated extraction of 0.5 M NaCl insoluble flour residue with water as the only solvent. It was not possible to further purify the two fractions without using denaturing solvents. As far as comparison of functionality is concerned, the purity of the protein fractions so obtained was considered satisfactory. It should be noted that there existed substantial compositional differences in both Gli-R and Glu-R fractions among the three cultivars, as indicated by electrophoretic results (Fig. 6.1 and 6.2).

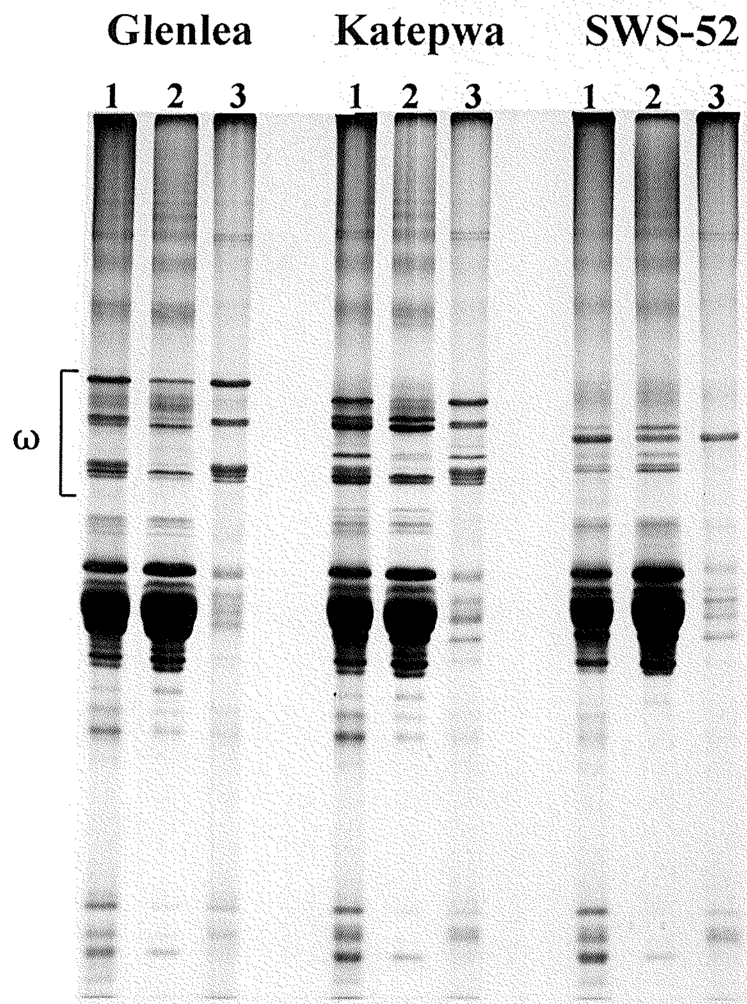
TABLE 6.1

Protein Contents and Composition of Gliadin-rich (Gli-R) and Glutenin-rich (Glu-R) Fractions Isolated from Glenlea, Katepwa and SWS-52 Flours[a]

	Glenlea		Katepwa		SWS-52	
	Gli-R	Glu-R	Gli-R	Glu-R	Gli-R	Glu-R
protein(%)	89.3±0.3	74.1±0.2	90.2±0.1	72.8±0.6	88.2±0.4	70.7±0.3
gliadin(%)	95.4±1.2	9.0±0.5	94.7±1.5	11.7±0.0	94.2±0.9	8.6±1.0
glutenin(%)	4.6±1.2	91.0±1.2	5.3±1.5	88.3±0.6	5.8±0.9	91.4±1.5

[a]mean of three replicates ± standard deviation

Fig. 6.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing and reducing conditions of protein fractions isolated from Glenlea, Katepwa and SWS-52 flours. 1, 0.5 M NaCl insoluble flour proteins (control); 2, gliadin-rich fraction; 3, glutenin-rich fraction. H, high molecular weight subunits of glutenin; ω, ω-gliadin.


UNREDUCED
Glenlea
Katepwa
SWS-52
1 2 3
1 2 3
1 2 3
ω

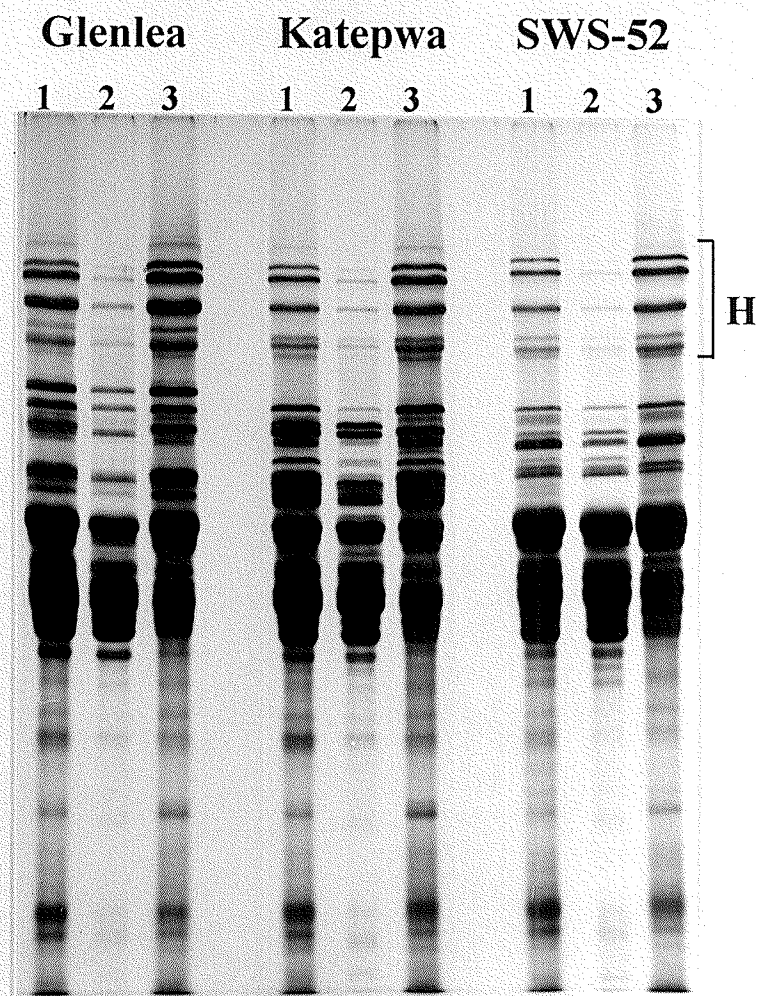
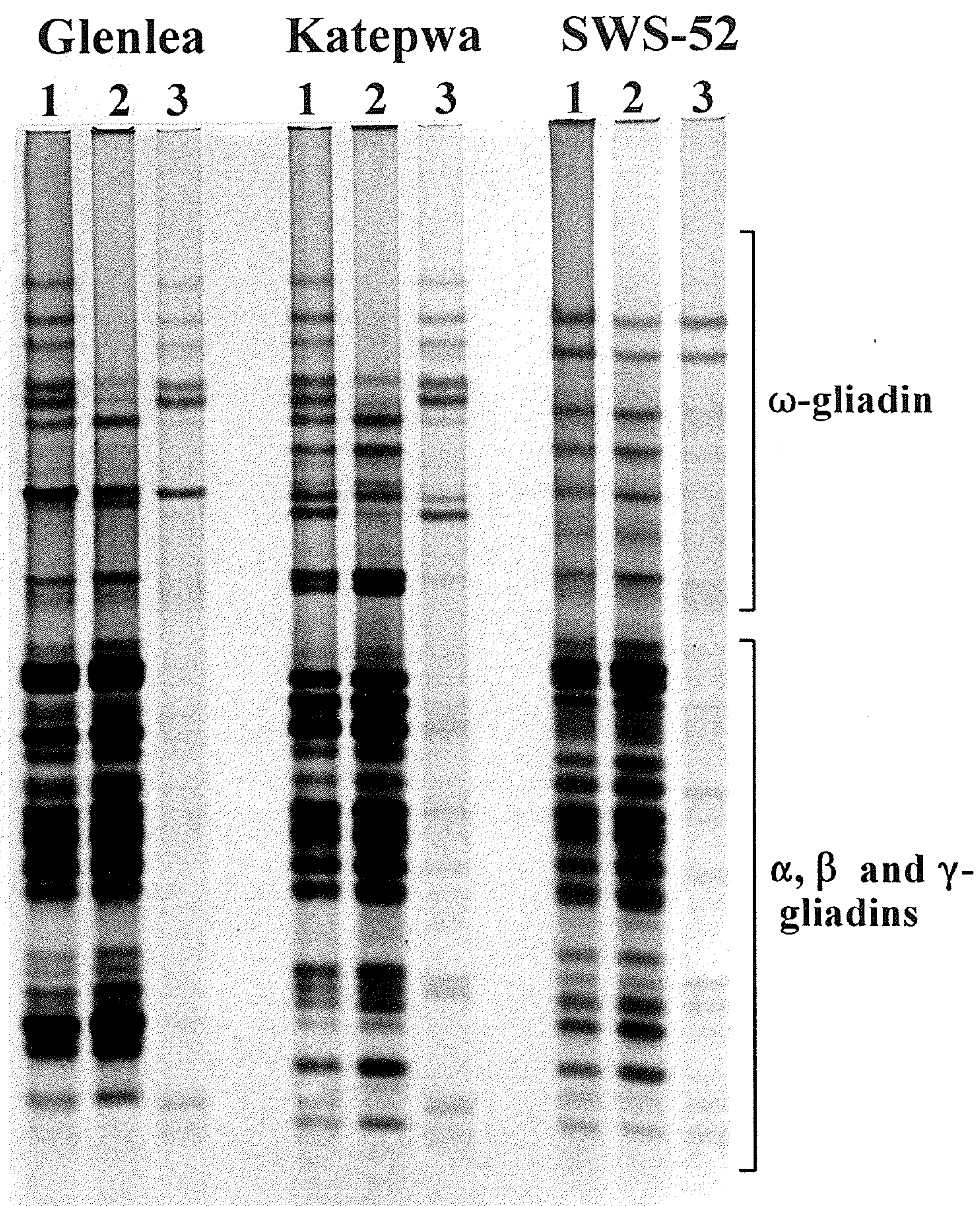

REDUCED
Glenlea
Katepwa
SWS-52
1 2 3
1 2 3
1 2 3
H

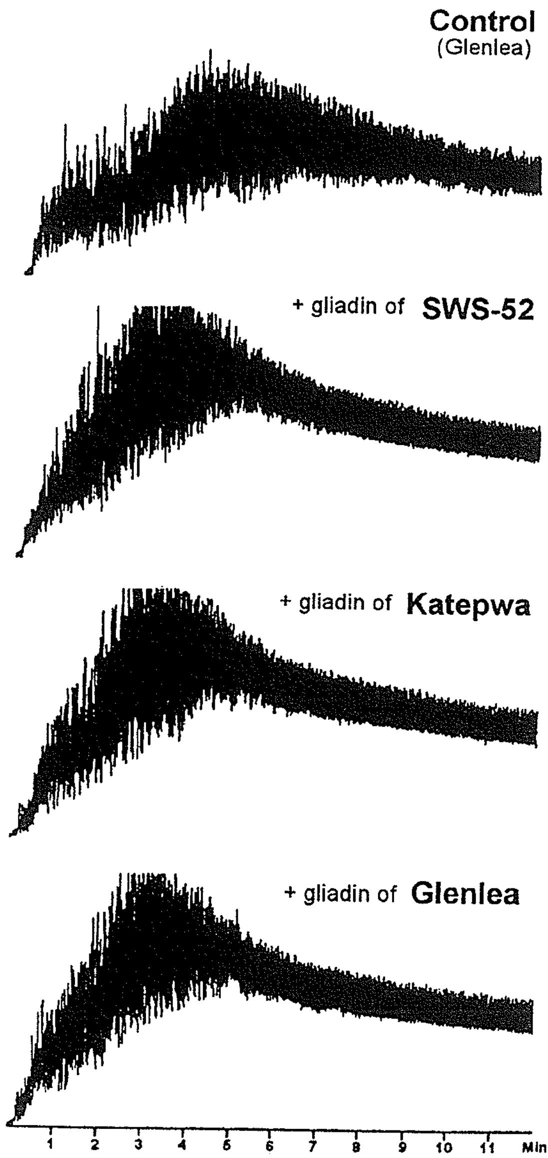
Fig. 6.2. Polyacrylamide gel electrophoresis at pH 3.1 of protein fractions isolated from Glenlea, Katepwa and SWS-52 flours as in Fig.1. 1, 0.5 M NaCl insoluble flour proteins (control); 2, gliadin-rich fraction; 3, glutenin-rich fraction.


Glenlea
Katepwa
SWS-52
1 2 3
1 2 3
1 2 3
ω-gliadin
α, β and γ-
gliadins

Effects of Gli-R and Glu-R Fractions on Dough Mixing

Effects of additions of varying amounts of Gli-R and Glu-R fractions on mixing properties of base flours were evaluated by 2-g mixograph. Typical mixograms for fortification at 2.5% protein level are shown in Fig. 6.3. Gli-R fractions of the three cultivars appeared to cause a similar weakening of the dough, while corresponding Glu-R fractions produced a strengthening effect that clearly varied with the source of the Glu-R fraction. This observation is consistent with the widely held view that the gliadin confers viscous properties on dough, while glutenin imparts strength and elasticity. Data computed from the mixograms (Fig. 6.4) confirmed that additions of the Gli-R fractions of the three cultivars produced similar effects on the MDT and WIP of Glenlea base flour at equivalent levels of protein enrichment. In contrast, the degree of dough-strengthening effects of the Glu-R fractions on MDT and WIP were cultivar dependent (Fig. 6.5). The Glu-R fraction isolated from Glenlea produced the greatest increase in MDT and WIP of the SWS-52 base flour. The dough-strengthening effect was lower for the Katepwa Glu-R fraction, and even lower still for SWS-52 glutenin. These results indicate that intercultivar differences in dough strength result from qualitative differences in glutenin.

Fig. 6.3. Computer generated mixograms of base flours (Glenlea and SWS-52) and base flours fortified with 2.5% gliadin-rich and glutenin-rich fractions isolated from Glenlea, Katepwa and SWS-52.


Control
(Glenlea)
+ gliadin of SWS-52
+ gliadin of Katepwa
+ gliadin of Glenlea
1 2 3 4 5 6 7 8 9 10 11 Min

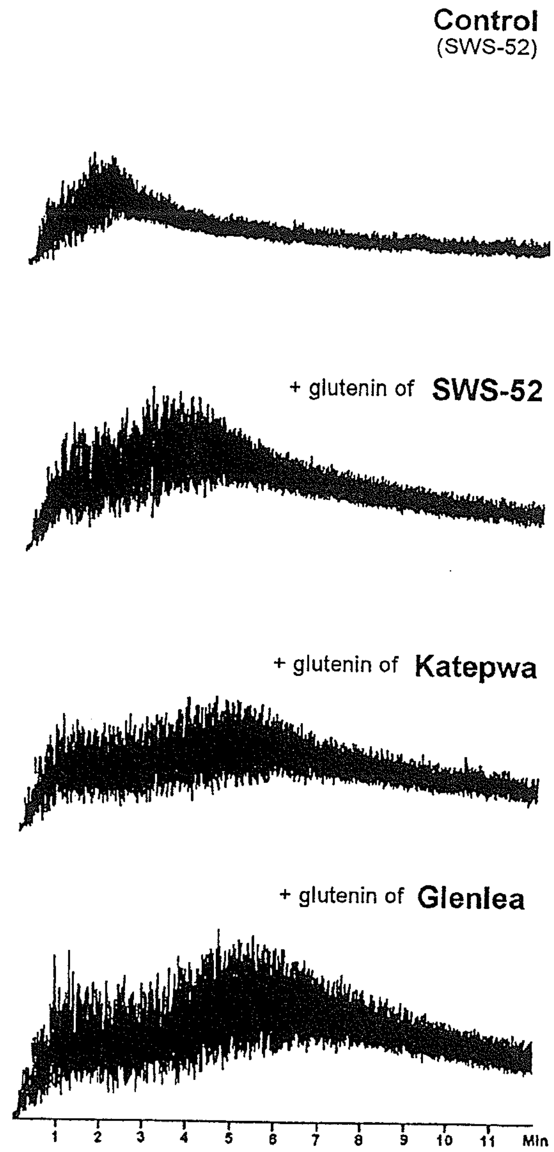
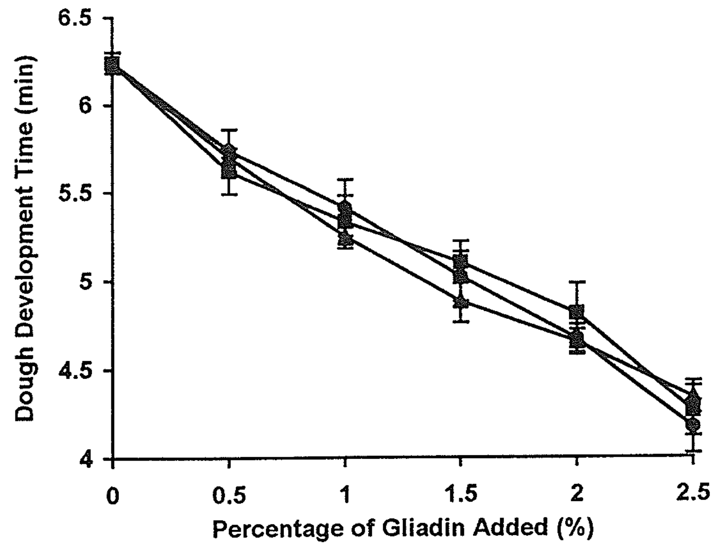
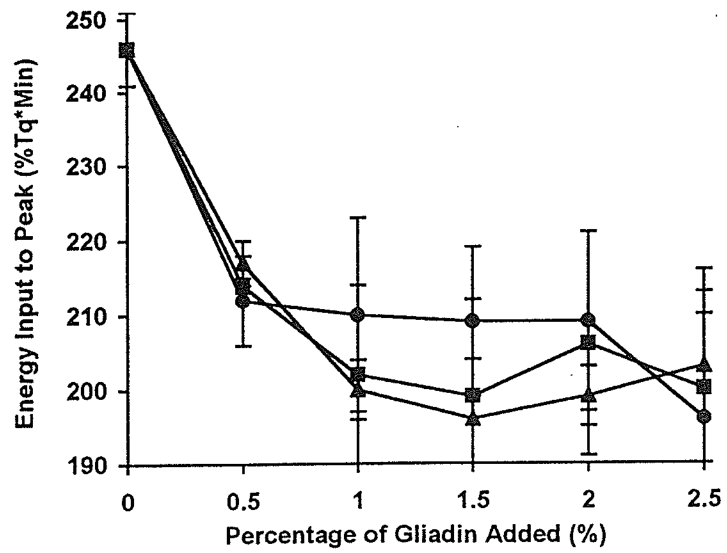

Control
(SWS-52)
+ glutenin of SWS-52
+ glutenin of Katepwa
+ glutenin of Glenlea
1 2 3 4 5 6 7 8 9 10 11 Min

Fig. 6.4. Effects on mixograph dough development time (MDT) and work input to peak (WIP) of additions of gliadin-rich fractions to Glenlea flour. The results are presented as the means of three replicates ± standard deviation. ---●---, Glenlea; ---■---, Katepwa; ---▲---, SWS-52. MDT and WIP values were not significantly different at any protein enrichment level.

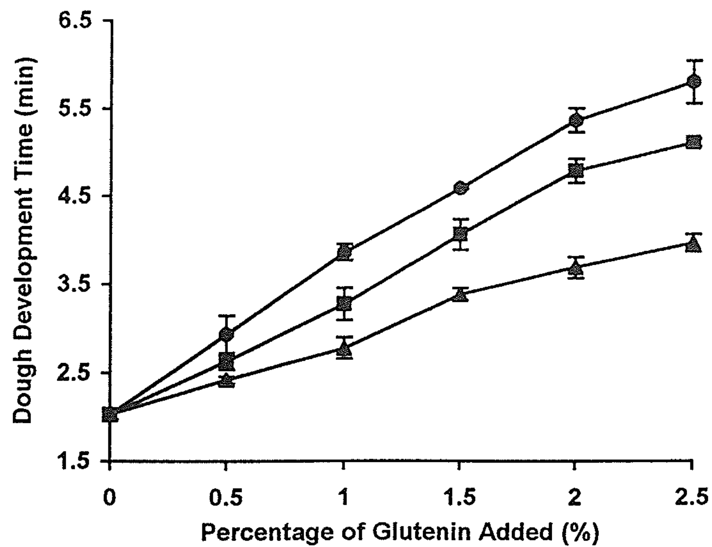


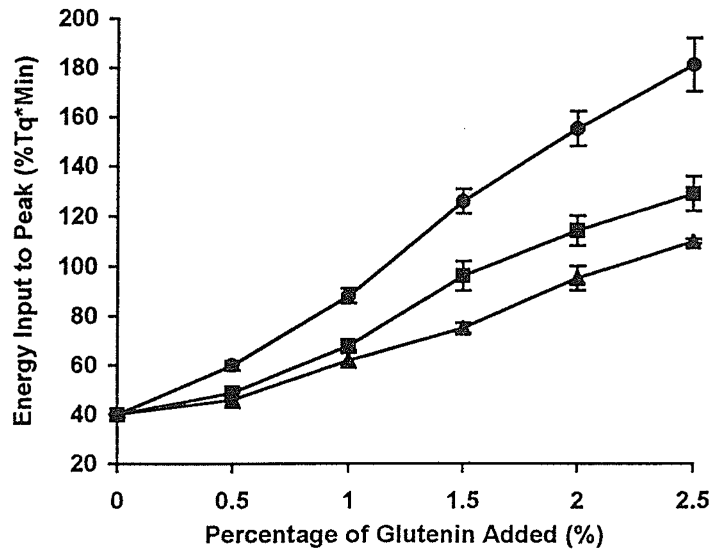
Dough Development Time (min)
6.5
6
5.5
5
4.5
4
0
0.5
1
1.5
2
2.5
Percentage of Gliadin Added (%)



Energy Input to Peak (%Tq*Min)
250
240
230
220
210
200
190
0
0.5
1
1.5
2
2.5
Percentage of Gliadin Added (%)

Fig. 6.5. Effects on mixograph dough development time (MDT) and work input to peak (WIP) of additions of glutenin-rich fractions to SWS-52 flour. The results are presented as the means of three replicates ± standard deviation. ---●---, Glenlea; ---■---, Katepwa; ---▲---, SWS-52. MDT and WIP for glutenin enrichment ≥ 1% were significant different ($p < 0.05$).


Dough Development Time (min)
6.5
5.5
4.5
3.5
2.5
1.5
0
0.5
1
1.5
2
2.5
Percentage of Glutenin Added (%)


Energy Input to Peak (%Tq*Min)
200
180
160
140
120
100
80
60
40
20
0
0.5
1
1.5
2
2.5
Percentage of Glutenin Added (%)

Discussion

One of the most direct ways to evaluate the functionality of protein fractions for breadmaking is by fortification of flour with isolated fractions (MacRitchie, 1987). This approach requires that the functionality of the proteins of interest are not altered by the isolation procedure used. In view of the labile nature of the gluten proteins, the solvents used and physical treatment should be as mild as possible to retain their functionality. The use of alcohol as extractant, which gives a relatively sharp separation of gliadin and glutenin, has been shown unsatisfactory for reconstitution studies, because the functional properties of the proteins are altered (Bloksma 1966; MacRitchie and Gras 1973; Booth and Melvin 1979; Chakraborty and Khan 1988a). Of the solvents most commonly used for wheat protein fractionation, dilute lactic, acetic and hydrochloric acids have been found to be relatively effective in reconstitution studies (Hoseney et al 1969a; Booth and Melvin 1979; MacRitchie 1978, 1985, 1987). However, changes in functional properties were observed if gluten proteins were left in acid solutions for any significant time (Goforth et al 1977; MacRitchie 1985). In order to limit exposure time in acid solutions, gluten has been dispersed in dilute HCl solutions by a high speed homogenizer in previous studies (MacRitchie 1985, 1987). Unfortunately, high-shear extraction may damage the native structure of the glutenin polymer (MacRitchie, 1975). In comparison with most previous fractionation studies, the procedure developed here using salt solution and water as the solvent for separation of gliadin and glutenin with magnetic stirring provides a very mild extraction environment along with an effective separation of functional gliadin and glutenin proteins.

Previous fractionation and reconstitution studies have established that differences in the quality of wheat flours for breadmaking reside mainly in the gluten proteins (Finney 1943; MacRitchie 1978). In regard to the specific protein fractions responsible for intercultivar differences in mixing and baking properties, conclusions arrived at by various reconstitution studies are somewhat contradictory. Some showed that the acid-insoluble glutenin fraction is responsible for the quality differences between cultivars (MacRitchie 1978, 1985; Booth and Melvin 1979); others that the acid-soluble glutenin governed dough strength (Preston and Tipples 1980); still others that gliadins control loaf volume potential of wheat flour (Hoseney et al 1969a, 1969b). These conflicting conclusions are most likely due to differences in the nature and purity of the fractions used, which can vary substantially depending on extraction procedures (Chakraborty and Khan, 1988a). Moreover, the heterogeneity of the fractions prepared in some reconstitution studies makes it invalid to use the terms gliadin and glutenin to describe them (Chakraborty and Khan, 1988a).

In the present study, clean separation of gliadin and glutenin was achieved. Fortification of base flours with flour protein fractions showed that the gliadin fractions from flours of widely different mixing properties behaved very similarly in their effects on dough mixing parameters obtained with the mixograph. Qualitative variation of gliadin did not contribute to intercultivar variations in dough mixing properties. In contrast, the effect of the glutenin fraction was genotype-dependent and accounted for the intercultivar differences in dough mixing properties. Results showed therefore, that for the flour samples examined, the key quality factor of wheat proteins resides in the glutenin

fraction. It seems plausible, from the dough mixing experiments carried out here, that this quality factor is the molecular size of native glutenin in wheat endosperm.

Chapter 7

EFFECT ON DOUGH MIXING PROPERTIES OF CHEMICAL INCORPORATION OF HIGH AND LOW MOLECULAR WEIGHT SUBUNIT FRACTIONS OF GLUTENIN ISOLATED FROM DIFFERENT WHEAT CULTIVARS

Abstract

Glutenin fractions comprising only high molecular weight (HMW) and low molecular weight (LMW) subunits were isolated from four Canadian wheat cultivars with diverse dough strength. The HMW- and LMW-glutenin subunit (GS) fractions (10 mg protein) were incorporated separately into doughs prepared from 2 g base flour using a reduction/oxidation procedure. Effects on the mixing properties of the doughs were evaluated with a direct drive computerized mixograph. Parameters evaluated were mixograph development time (MDT), work input to peak (WIP), mixogram peak height (MPH) and peak band width (PBW). The results indicated that incorporation of LMW-GS from different wheat cultivars had relatively marginal effects on the dough mixing properties of the base flour. In contrast, incorporation of HMW-GS resulted in a substantially significant increase in dough strength. For MDT, WIP, MPH and PBW, the average increases were 29, 51, 16 and 27%, respectively. There were no significant differences in dough-strengthening effects for the HMW-GS isolates of different cultivars. Accordingly, the intercultivar variation in dough mixing properties of the different flours appears to be due to variation in the concentration of the HMW-GS fraction rather than in the qualitative variation of subunits.

Introduction

Much of the interest during the last 15 years in the molecular basis of breadmaking quality has focused on the variation and genetic control of glutenin subunits and their association with wheat end-use quality (Shewry et al 1992). Currently available evidence indicates that both qualitative and quantitative differences in HMW- and LMW-GS appear to contribute to intercultivar variation in breadmaking quality of wheat flour (Kolster and Vereijken 1994; Gupta et al 1995 and references therein). Apart from reconstitution studies which have provided direct information on the relative importance of glutenin in breadmaking quality (MacRitchie 1978, 1985, 1987; MacRitchie et al 1991), knowledge about the functional role of specific groups of wheat proteins has been mainly based on correlative studies, in which the proportion of a protein fraction in flours of different quality is determined and statistically correlated with end-use quality.

Recently, a technique for chemical incorporation of glutenin subunits into wheat dough by partial reduction followed by oxidation has been developed by Bekes et al (1994a). This technique has been applied to study the effects on dough mixing properties of incorporation of individual and pairs of HMW-GS (Bekes and Gras 1994, Bekes et al 1994b, Bekes et al 1995). There have been no reports on the application of this chemical incorporation procedure using protein fractions comprising total HMW- or LMW-GS. It was previously shown by fractionation/fortification (Chapter 6) that differences in dough mixing requirements among cultivars were due to qualitative differences in the glutenin fraction. The aim of this study was to follow up those results using Bekes' reduction/oxidation procedure to attempt to elucidate the precise qualitative factor(s) in

glutenin responsible for the differences found. In this regard, the effects on dough mixing of full complements of HMW- and LMW-GS fractions isolated from different wheat cultivars were separately investigated.

Materials and Methods

Flour Samples

Four flours with diverse dough mixing properties, milled from wheat cultivars Glenlea, Roblin, Katepwa and AC Karma, were selected for the preparation of HMW- and LMW-GS fractions. Pertinent milling details and the technological properties of the flours have been described in Table 4.1 of Chapter 4. Flour of AC Karma was selected as the base flour for incorporation of added glutenin subunits by reduction/oxidation.

Preparation of Total HMW- and LMW-GS Fractions

The method used to prepare the HMW- and LMW-GS fractions was adapted from the procedure described by Marchylo et al (1989). Flour samples (20.0 g) were extracted three times (1 h, 0.5 h and 0.5 h, respectively) with 50% 1-propanol (200 ml) at room temperature (23 ± 1°C), with centrifugation at 1,000*g* for 10 min between each extraction. The supernatants were discarded. The residue, which is free of monomeric proteins, was extracted twice (1 h each) with 50% 1-propanol containing 0.2% (w/v) dithiothreitol (DTT) (100 ml) at room temperature and centrifugation at 10,000*g* for 10 min. The pooled supernatants were adjusted to 62.5% (v/v) 1-propanol and 0.2% DTT and left to stand for 1 h. After centrifugation (10,000*g*, 10 min), the resulting supernatant and pellet

containing HMW- and LMW-GS, respectively, were collected and dialysed against 0.1% (v/v) acetic acid at 4°C for 72 h and freeze-dried. The protein contents of the freeze-dried materials were determined by micro-Kjeldahl method (AACC 1983).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Ng and Bushuk (1987) except that the separating gel concentration was altered to 14%.

Dough Mixing and Chemical Incorporation of Glutenin Subunits

The reduction/oxidation procedure, developed by Bekes et al (1994a), for incorporating added glutenin subunits into dough, was used in conjunction with a computerized 2 g mixograph (National Manufacturing Division, TMCO, Lincon, NE). Flour (2.0 g, 14% m.b.) was supplemented with 10 mg of HMW- or 10 mg LMW-GS, and mixed with 1.0 ml water plus 0.1 ml water containing 5 μg DTT for 30 s. The mixture was allowed to react for 4 min without mixing. The partially reduced doughs were treated with the oxidant solution (0.1 ml containing 20 μg potassium iodate). Mixing was continued for 30 s, the dough was allowed to react for 5 min, and then it was mixed for a further 12 min. The same protocol was followed 1) for the control treatment, i.e. doughs manipulated by the same reduction/oxidation procedure but without addition of any glutenin subunits, and 2) for a blank treatment, in which water was added without reductant, oxidant and glutenin subunits. Dough mixing parameters were computed automatically using the accompanying Mixsmart software. Parameters

determined were mixing time to peak dough development (MDT), work input to peak (WIP), mixogram peak height (MPH), and peak band width (PBW). Dough mixing was performed in duplicate and mean results are reported.

Results

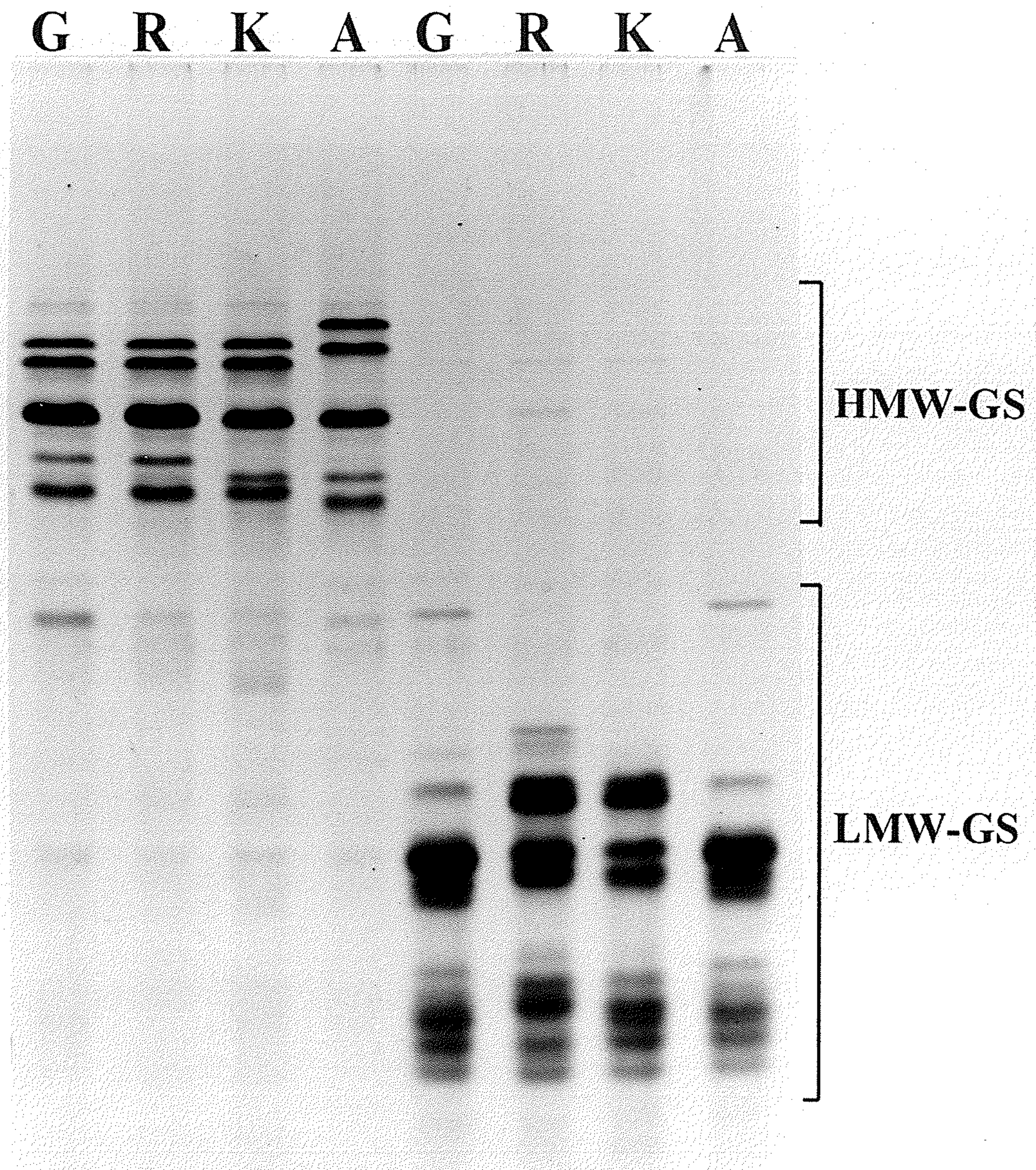
Isolation of Total HMW- and LMW-GS Fractions

SDS-PAGE analysis (Fig. 7.1) demonstrated that fractionation of HMW- and LMW-GS isolated from 50% 1-propanol insoluble residue was very effective with essentially no cross contamination. HMW-GS compositions according to the nomenclature of Payne and Lawrence (1983) and Marchylo et al (1992) were as follows: Glenlea and Roblin, 2*, 7+8, 5+10; Katepwa, 2*, 7*+9, 5+10; and AC Karma, 1, 7*+9, 2+12. The protein contents (as is basis) of the HMW- and LMW-GS fractions, respectively, were as follows: Glenlea, 92.9±1.1% and 88.6±1.3%; Roblin 93.7±1.9% and 86.6±1.2%; Katepwa 93.5±1.6% and 84.5±0.8%; AC Karma 92.4±1.4% and 86.2±1.6%. The experimental error indicated is based on three determinations.

Effects of Incorporation of HMW- or LMW-GS Fractions on Dough Mixing Properties

The validity of the reduction/oxidation procedure on the chemical incorporation of glutenin subunits was first evaluated using a base flour alone to answer the following question, i.e. do doughs from flours subjected to the reduction/oxidation process have the same mixing properties as doughs from flour without reduction/oxidation? Accordingly, the mixing properties of the doughs subjected to control and blank

Fig. 7.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of total high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits (GS) isolated from four wheat cultivars. G, Glenlea; R, Roblin; K, Katepwa; and A, AC Karma.


G
R
K
A
G
R
K
A
HMW-GS
LMW-GS

treatments were compared. Results showed that the mixing properties of the control treatment were essentially identical to that of the blank treatment. The MDP and WIP for the control treatment were 1.08±0.01 min and 35.8±3.0 %Tq*min (arbitrary unit), whereas for blank treatment, the corresponding values were 1.07±0.02 min and 35.5±1.9 %Tq*min, respectively. These results are in agreement with those obtained by Bekes et al (1994a) that by careful selection of reaction conditions, the mixing properties of a partially reduced dough can be fully restored by oxidation.

Figure 7.2 shows typical mixograms of the control dough and the control doughs with 10 mg of chemically incorporated total HMW- or LMW-GS isolated from different wheat cultivars. The results (Table 7.1) indicated that incorporation of LMW-GS from different wheat cultivars had relatively marginal effects on the dough mixing properties of the base flour. Only Glenlea and Roblin LMW-GS fractions showed statistically significant effects; MDT for Glenlea LMW-GS was significantly greater, and MPH for both Glenlea and Roblin was significantly lower. In contrast, incorporation of HMW-GS resulted in a substantially significant increase in dough strength. For MDT, WIP, MPH and PBW, the average increases were 29, 51, 16 and 27%, respectively. Qualitative differences in HMW-GS composition among cultivars Glenlea (or Roblin) and Katepwa or AC Karma (e.g. HMW-GS 5+10 versus 2+12) did not result in discernable differences in dough properties, i.e. there were no differences in dough-strengthening effects for the HMW-GS isolates of different cultivars. Figure 7.3 graphically illustrates the effects of chemical incorporation of LMW- and HMW-GS fractions on MDT and WIP values relative to the control treatment.

Fig. 7.2. Computer generated mixograms of the control dough manipulated by the reduction/oxidation procedure and the control doughs augmented with 10 mg of total high molecular weight (HMW) or low molecular weight (LMW) glutenin subunits (GS) isolated from different wheat genotypes using the reduction/oxidation procedure.

Control

+ LMW-GS of **AC Karma**

+ LMW-GS of **Katepwa**

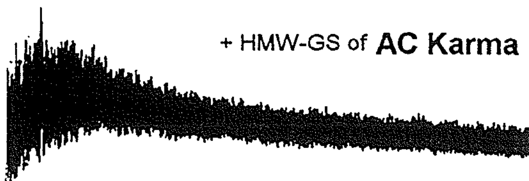
+ LMW-GS of **Roblin**

+ LMW-GS of **Glenlea**

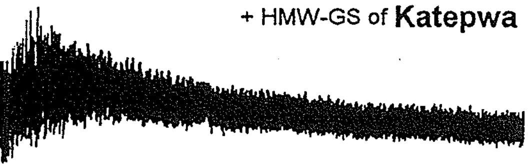
1 2 3 4 5 6 7 8 9 10 11 Min

Control

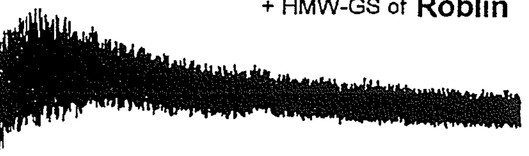
+ HMW-GS of **AC Karma**



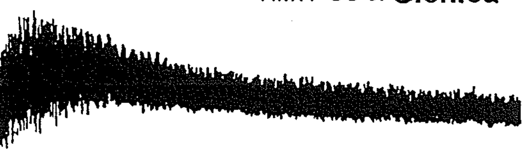
+ HMW-GS of **Katepwa**



+ HMW-GS of **Roblin**



+ HMW-GS of **Glenlea**



1 2 3 4 5 6 7 8 9 10 11 Min

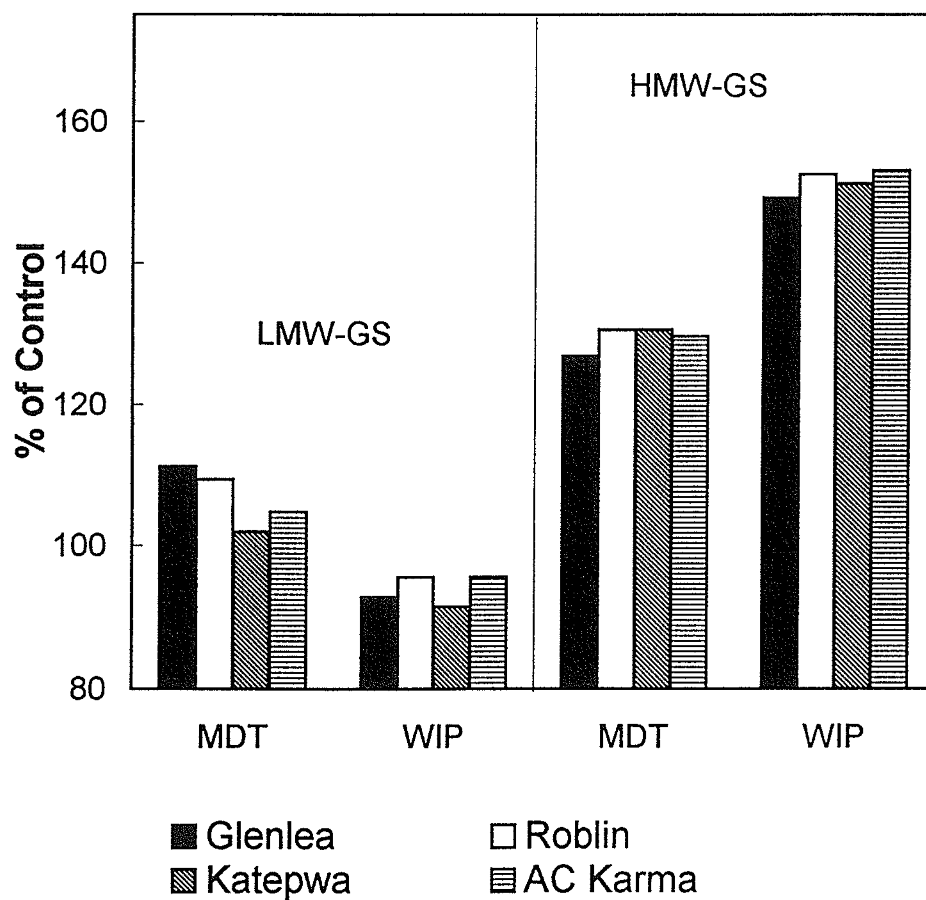
TABLE 7.1

The Effects on Mixing Parameters of Incorporation of Total High Molecular Weight (HMW) and Low Molecular Weight (LMW)-Glutenin Subunits (GS) Isolated from Different Wheat Cultivars into Base Flour Doughs[a,b]

	LMW-GS				HMW-GS			
	MDT[c]	WIP[d]	MPH[e]	PBW[f]	MDT	PWI	MPH	PBW
Control	1.08±0.01a	35.8±3.0a	39.2±2.7c	21.5±2.0b	1.08±0.01a	35.8±3.0a	39.2±2.7a	21.5±2.0a
Glenlea	1.20±0.01b	33.2±0.3a	32.3±0.5a	16.8±0.4a	1.37±0.01b	53.4±2.2b	46.1±1.7b	28.7±1.6b
Roblin	1.18±0.08ab	34.2±1.0a	33.8±1.3ab	17.8±0.7ab	1.41±0.07b	54.6±3.7b	45.7±1.2b	27.6±0.6b
Katepwa	1.10±0.03a	32.7±0.1a	34.9±0.9cb	18.3±0.3b	1.41±0.03b	54.1±1.7b	44.6±0.1b	26.4±0.4b
AC Karma	1.13±0.04ab	34.2±2.4a	35.4±1.0cb	19.7±2.2ab	1.40±0.06b	54.8±3.4b	46.0±0.7b	26.5±0.6b

[a] Means of duplicates ± standard deviation
[b] Values with the same letter are not significantly different (α=0.05)
[c] Mixing time to peak (min); [d] Work input to peak (%Tq*min); [e] Mixogram peak height (arbitrary units); [f] Peak band width at peak (arbitrary units).

Fig. 7.3 Effect on mixograph dough development time (MDT) and work input to peak (WIP) of chemical incorporation of total low molecular weight (LMW)- and high molecular weight (HMW)-glutenin subunits (GS) isolated from different wheat cultivars.



HMW-GS
LMW-GS
% of Control
160
140
120
100
80
MDT
WIP
MDT
WIP
Glenlea
Roblin
Katepwa
AC Karma

Discussion

The results obtained in this study confirmed the importance of HMW-GS in determining dough strength. The HMW subunits have been considered to form the backbone of the glutenin polymer (Shewry et al 1992). With respect to the qualitative variation, the present study did not reveal any functional differences between the different combinations of HMW-GS of cultivars of diverse dough strength. Recently, Schropp et al (1995) and Schropp and Wieser (1996) reported that different combinations of HMW-GS (5, 7, 9, 10 versus 2, 6, 8, 12) did not influence the kinetics of oxidation and polymerization. The effect of reoxidised HMW subunits on gluten rheological properties did not depend on the HMW-GS composition. Results of this study are also in agreement with those of Lawrence et al (1988), who showed that mixtures of different HMW-GS were similar in terms of their effect on quality. Lawrence et al (1988) concluded that different gene loci effects are due to their ability to produce different amounts of HMW-GS. In contrast, Bekes and Gras (1994) showed that incorporation of pairs of subunits 5+10 had a larger dough-strengthening effect than the same amount of subunits 2+12 which appears to be consistent with genetic evidence for the correlation between specific HMW subunit alleles and breadmaking quality (Payne 1987). Bekes and Gras (1994) concluded that the size of a subunit seems to be the important property that determines its effect on mixing properties; the larger the subunit that is incorporated, the longer the mixing time to peak development.

The reason for no differential mixing effects reflecting the polymerisation of different total HMW-GS in our study in contrast to the results of Bekes and co-workers

may be due to reoxidation kinetics associated with number and type of subunits that are polymerised. Szabo et al (1995) showed that pairwise mixtures of x-type and y-type HMW-GS oxidise significantly faster than either type alone. Additionally, differences in dough strengthening effects for reoxidation of mixtures of subunits 5+10 and 2+12 were relatively small compared to the differences that were observed between individual x- and y-type subunits (Bekes et al 1995). Accordingly, it is plausible that reoxidation of the full complement of x- and y-type subunits in total HMW-GS of different genotypes results in uniformly large polymers and comparable dough mixing effects reflecting total HMW-GS concentration only.

The role of qualitative differences in the LMW-GS in baking quality has not been extensively studied. Using genetic lines lacking one, two or all three *Glu-3* loci, Gupta et al (1995) showed that LMW-GS contributed positively to dough strength, although far less than the HMW subunits. In contrast, results of the present study showed that the incorporation of LMW-GS from diverse wheat cultivars had no definitive effect on the dough mixing properties. It should be noted that complete incorporation of 10 mg of HMW-GS could result in about a 50% increase of total HMW-GS of base flour, while only approximately a 20% increase in LMW-GS content was theoretically achieved after incorporation of same amount of LMW-GS. Experiments with higher levels of LMW-GS enrichment need to be carried out to further explore the functionality of LMW-GS. Nevertheless, results indicate that the intrinsic polymerization ability of HMW-GS (and possibly the inherent elasticity) is very different from that of the LMW-GS. On the same protein basis, HMW-GS had a far greater effect than the LMW-GS on dough strength.

Chapter 8

SALT-INDUCED DISAGGREGATION/SOLUBILIZATION OF GLUTEN PROTEINS IN WATER[1]

Abstract

The effect of salt concentration used in preparing gluten, on the subsequent dissolution of gluten in water was examined. Flour from a Canadian hard red spring wheat cultivar Katepwa was used to prepare glutens using three different solvents, i.e. distilled deionized water (DDW), 0.2% NaCl solution and 2% NaCl solution. The isolated wet glutens were sequentially extracted with DDW, providing four water soluble fractions and the insoluble residue. The amount of protein in each fraction was determined and respective compositions were assessed electrophoretically under reducing and non-reducing conditions. Surprisingly, DDW extracts of gluten prepared with 2% NaCl contained almost all of the gliadins except some ω-gliadin components, and most of the polymeric glutenin. For the gluten prepared with 0.2% NaCl, most of the gliadin but a small portion of glutenin was extracted. For gluten prepared with DDW, only part of the gliadins and almost no glutenin was extractable with water. The DDW solubility of gluten proteins prepared in DDW, 0.2% NaCl and 2% NaCl were 27, 52, and 85%, respectively after four sequential extracts with DDW. The large increases in the gliadin and glutenin solubility in DDW when the gluten is prepared in salt solution (after removal of most of the salt) can be explained on the basis of a salt-induced conformational change of the proteins which renders water a more effective solvent.

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[1]Published in Journal of Cereal Science 24:241-246 (1996) by B.X. Fu, H.D. Sapirstein and W. Bushuk

Introduction

The limited solubility of gliadin and glutenin in water is an important factor in the uniqueness of wheat for breadmaking. It has been shown that gliadins are soluble in aqueous media only at very low ionic strength (Mills 1954, Holme and Briggs 1959). The poor solubility of glutenin in aqueous solvents has been generally attributed to its large molecular size and intermolecular aggregation arising from strong noncovalent interactions involving hydrogen and hydrophobic bonds (MacRitchie 1992, Weegels et al 1994b). As with the gliadins, the relative scarcity of basic amino acids in glutenin (Wrigley and Bietz 1988) with positively charged side chains at the slightly acidic pH of normal gluten, and ionizable groups in general, are important factors in accentuating the potential role of ionic interactions and salt effects in the solubility of these proteins in aqueous solvents. Previous work on salt effects has focused on the effects of salt on dough rheology and the extractability of gluten proteins in various organic solvents (Kim and Bushuk 1995 and references therein). In contrast, very little information has been reported on the effects of prior salt treatments on gluten protein solubility in water (Clements 1973). In that study, gluten that was homogenized in 1M NaCl was repeatedly and exhaustively extracted with deionized water. It was reported that up to 90% of the gluten proteins were extracted (60% in initial extracts plus 30% upon exhaustive washing), although the identity of these proteins was not determined.

In preliminary experiments related to this thesis research, a large increase in the solubility of gliadin and glutenin in water was observed when the parent gluten was prepared in the presence of salt; the higher the salt concentration, the higher the

solubility. This Chapter reports these new findings and advances a new hypothesis on a possible role of charged groups in the functionality of gliadin and glutenin in wheat flour doughs.

Materials and Methods

The flour used was milled on a Buhler experimental mill from grain of the Canadian hard red spring wheat cultivar Katepwa. Its protein and ash contents were 13.3% and 0.45% (14% moisture basis), respectively.

Wet glutens were prepared from the flour by washing in a Glutomatic gluten washer (Falling Number AB, Sweden) at room temperature (23±1°C) using three different solutions, distilled deionized water (DDW), 0.2% NaCl solution, and 2% NaCl solution. Flour (10 g) was mixed with 5.0 ml of washing solution for 15 seconds and washed under a stream of washing solution (60 ml/min) for 5 min. Both the dough formation and subsequent gluten washing were automatically carried out by the Glutomatic system. The gluten balls prepared by DDW and 0.2% NaCl were immediately cut into small pieces (approximately 50 mg) and subjected to four sequential extractions with 50 ml DDW for 1 h at room temperature (23±1°C) with magnetic stirring. After each extraction, the supernatant obtained by centrifugation (10,000g, 15 min) was decanted. Thus, four soluble fractions and an insoluble residue were obtained. The gluten ball prepared by 2% NaCl was first washed in the gluten washer for 2 min with 0.2% NaCl to remove most of the residual salt in the gluten. The reason for using 0.2% NaCl instead of DDW was to maintain the insolubility of the gluten proteins at this stage. The partially desalted

gluten was extracted four times with DDW as described above.

The three glutens and their fractions were freeze-dried. Protein contents of the dry preparations were determined by the micro-Kjeldahl method. Subsamples of the original glutens and the fractions were analyzed by polyacrylamide gel electrophoresis in acidic conditions (APAGE) (Sapirstein and Bushuk 1985) and by sodium dodecyl sulfate (SDS)-PAGE (Ng and Bushuk 1987). Freeze-dried glutens (20 mg) were extracted three times with 1 ml of aqueous 70% ethanol for 30 min at room temperature in a micro-centrifuge tube to determine the solubility of original glutens in this solvent. The protein contents of the three glutens and related fractions were determined by the micro-Kjeldahl method.

Results

Protein content, recovery and solubility in 70% (v/v) ethanol of the three glutens are compared in Table 8.1. The protein contents for all three glutens were comparable and ranged from 82% for DDW gluten to 86% for 2% NaCl gluten. The protein recovery of gluten prepared using 2% NaCl (75% of flour protein) was significantly lower than for gluten prepared using 0.2% NaCl or DDW (81-82%). This was due to the extra two min washing of the 2% NaCl gluten with 0.2% NaCl to remove residual salt; protein was found in this extra washing solution. The DDW gluten contained significantly less 70% ethanol soluble protein than the glutens prepared by the salt solutions. Gliadins are soluble in water of low ionic strength and are washed out during gluten washing by DDW. The appreciable swelling of glutens isolated with salt solution during extraction may also increase their solubility in 70% ethanol. Glutens prepared with salt solutions

appeared to be more elastic than those prepared using DDW. The APAGE and SDS-PAGE patterns (see below) indicated that the three glutens were similar on the basis of subjectively assessed band intensity and qualitatively identical in protein composition (compare lanes G in Fig. 8.1 or Fig. 8.2).

Table 8.2 shows that the extractability of protein from the wet gluten by DDW increased substantially when the gluten was prepared with the salt solutions compared with the gluten prepared by DDW. The percentage of gluten protein extracted by the four DDW extractions was 27.5% for the DDW gluten, 51.7% for the 0.2% NaCl gluten and 84.5% for the 2% NaCl gluten. Although DDW gluten originally contains slightly less gliadin content, that difference cannot account for the large increase of protein soluble in DDW of glutens prepared by salt solutions. The observation that the 0.2% NaCl prepared gluten swelled more during the second extraction than it did in the first explains why the second extract of the 0.2% NaCl gluten yielded significantly more protein (by 7%) than the first extract.

APAGE patterns (Fig. 8.1) showed that more gliadin (judged by band intensity) was extracted from the glutens prepared by salt solutions than from the gluten prepared by DDW. In fact only some ω-gliadins (mainly the slow moving components) and trace amounts of α-, β- and γ-gliadins remained in the DDW insoluble residue from gluten prepared by 2% NaCl. In contrast, the gliadin pattern of the residue of the gluten washed out with DDW was essentially the same as that of unfractionated gluten. That some ω-gliadins were more difficult to extract with DDW than other gliadins is probably due to their lower ionic and more non-polar character (Kasarda et al 1976) and/or specific

interaction with glutenin (Kasarda 1989). Why the slow-moving ω-gliadins are differentiated in this way is unclear. A differentiation between the slow and fast migrating ω-gliadins in terms of their solubility in 50% and 70% aqueous 1-propanol was previously found (see Chapter 3); the slow moving ω-gliadins tended to be soluble in both 50% and 70% 1-propanol, whereas the fast moving ω-gliadins were insoluble (along with glutenin) in 70% 1-propanol. This indicates that the slow-moving components are more hydrophobic as they are soluble in a higher concentration of propanol.

SDS-PAGE patterns of the glutens and their fractions after reduction with 2-mercaptoethanol (Fig. 8.2) clearly showed the presence of HMW subunits of glutenin in all the DDW-soluble fractions of the gluten prepared with 2% NaCl but only very faint bands in the lanes for fractions of DDW gluten. Comparison of the pattern of the four extracts and that of the residue (Fig. 8.2, lane R) showed that essentially all of the glutenin in the 2% NaCl gluten was extracted by DDW whereas very little was extracted from the DDW gluten or gluten washed in 0.2% NaCl. SDS-PAGE of these extracts under non-reducing conditions (not shown) did not show any evidence of HMW subunits of glutenin. Thus the extractability of glutenin after treatment of gluten with 2% NaCl for example, was not accompanied by any depolymerization of glutenin into subunits, or oligomers small enough to enter the gel, during the sequential extraction with DDW.

Table 8.1

Protein Composition of Three Glutens Prepared Using Different Solutions[a]

Glutens	Protein Content (%)[b]	Flour Protein Recovery (%)[c]	Solubility in 70% Ethanol[d]	
			Soluble	Insoluble
DDW gluten	82.4±0.3	81.2±0.5	48.7±1.4	51.3±1.8
0.2% NaCl gluten	82.7±0.6	82.0±1.4	53.6±2.2	46.3±1.5
2.0% NaCl gluten	85.9±1.1	75.3±0.9	52.5±2.9	47.5±2.3

[a] Means of three replicates±standard deviation.
[b] As is basis after freeze-drying.
[c] Gluten protein as percentage of flour protein.
[d] Percent of 70% ethanol soluble and insoluble proteins are expressed as a percentage of the sum of the two fractions.

Table 8.2

Solubility (%) in Distilled and Deionized Water (DDW) of Proteins from Katepwa Glutens Prepared by DDW and Salt Solutions[a]

Glutens	1st extract	2nd extract	3rd extract	4th extract	residue	protein recovery
DDW gluten	12.1±0.3	9.6±0.4	4.0±0.2	1.8±0.2	72.5±0.7	98.5±1.5
0.2% NaCl gluten	19.6±0.8	26.7±0.5	4.2±0.3	1.1±0.1	48.3±0.5	96.7±0.2
2.0% NaCl gluten	39.0±0.3	32.0±1.9	9.7±0.6	3.9±0.3	15.5±1.6	98.6±1.8

[a] percent of the sum of the proteins in all fractions ± standard deviation.

Fig. 8.1. Polyacrylamide gel electrophoresis at pH 3.1 of the 70% (v/v) ethanol soluble gliadins of the four sequential water soluble fractions (1 - 4) and insoluble residue (R) from glutens isolated with distilled and deionized water (DDW gluten), 0.2% NaCl solution (0.2% NaCl gluten) and 2% NaCl solution (2% NaCl gluten). The unfractionated glutens (G) were analyzed as controls.

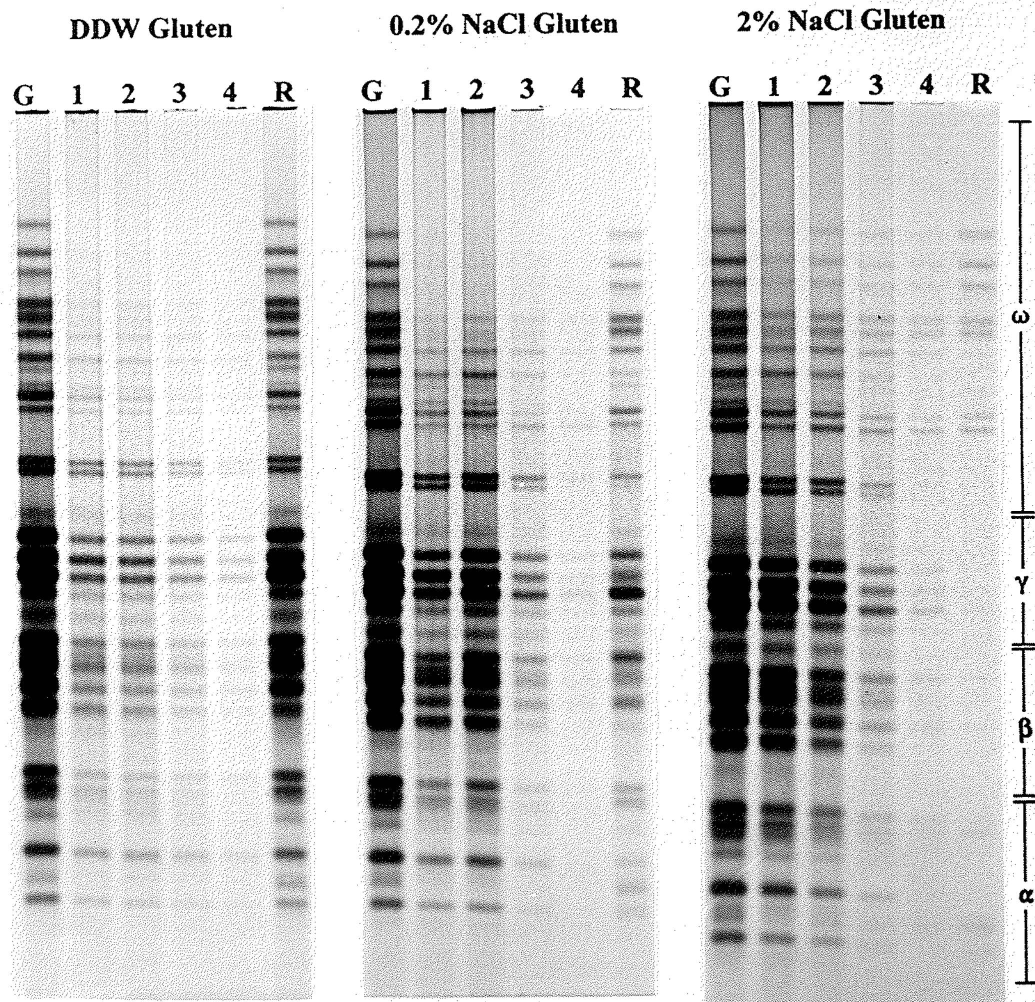
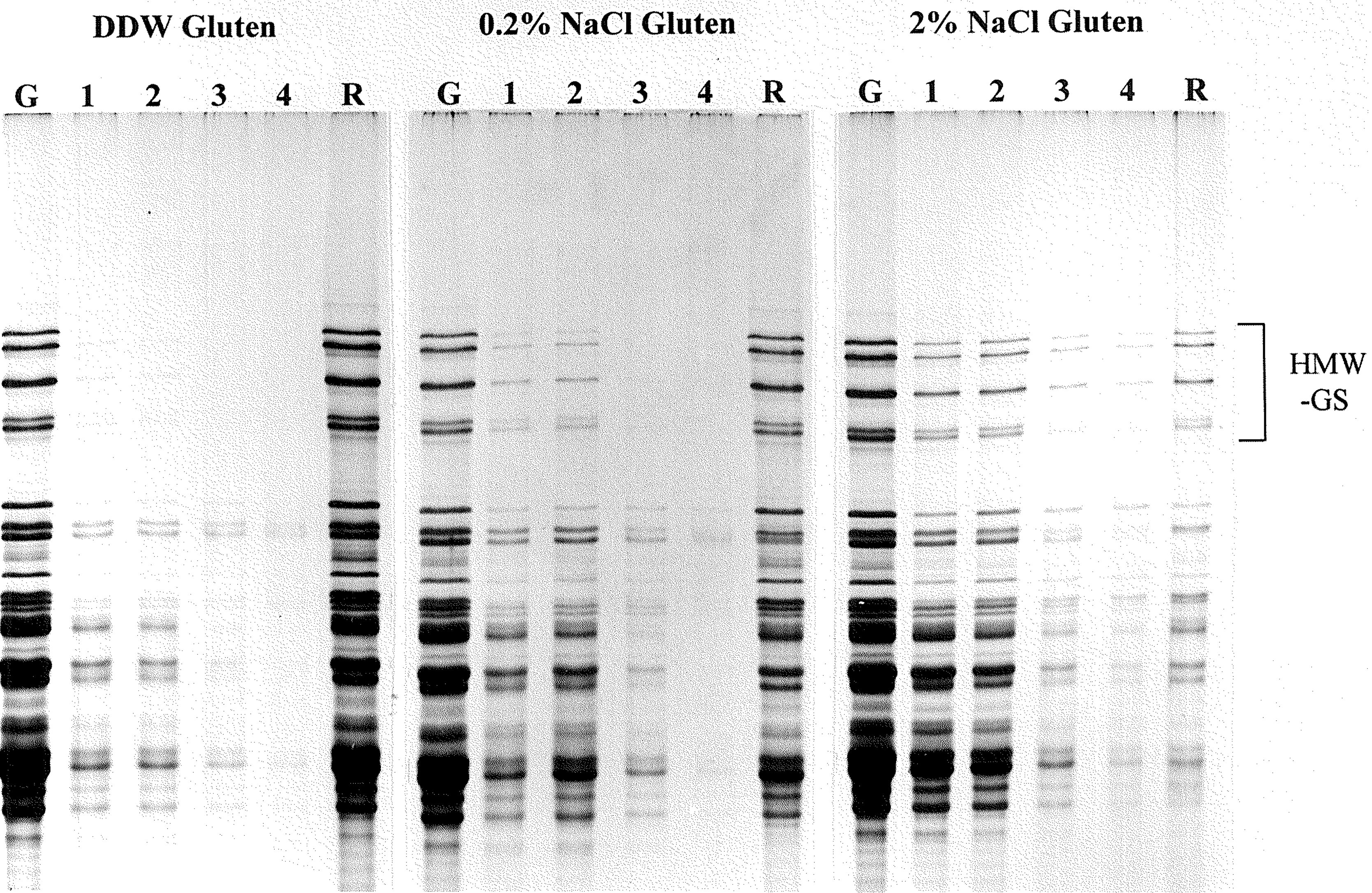

DDW Gluten
0.2% NaCl Gluten
2% NaCl Gluten
G 1 2 3 4 R
G 1 2 3 4 R
G 1 2 3 4 R
ω
γ
β
α

Fig. 8.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reduced four sequential water soluble fractions (1 - 4) and insoluble residue (R) from glutens as in Fig. 8.1. The reduced unfractionated glutens (G) were analyzed as controls. High molecular weight glutenin subunits are abbreviated as HMW-GS.


DDW Gluten
0.2% NaCl Gluten
2% NaCl Gluten
G 1 2 3 4 R
G 1 2 3 4 R
G 1 2 3 4 R
HMW
-GS

Discussion

The large increases in the solubility of gliadin and glutenin in DDW when the gluten is prepared in salt solution (after removal of most of the salt) can be explained on the basis of a salt-induced conformational change of the proteins which renders them more soluble in water. Clements (1973) speculated that the conformational change in gluten proteins, leading to increased dispersibility in water, was the breaking of ionic bonds and the exposure of polar groups when gluten was subjected to aqueous media of high ionic strength. Such a hypothesis may only partly explain the solubility phenomenon we observed in this study. The presence of the relatively non-chaotropic salt NaCl, by increasing water structure, would result in a general increase in the strength of hydrogen bonding within and among polypeptide chains of the protein molecules as water molecules are drawn away from these structures to interact with sodium and chloride ions. Presumably, the resulting increase in intra- and inter-protein interactions would result in a compaction of the gluten structure as envisioned by Galal et al (1978). This would account for the well-known dough strengthening effect of NaCl (Hlynka 1962).

From the results presented, it seems clear that the native conformation of gluten proteins is altered significantly upon the addition of salt. It also seems plausible that upon subsequent removal of the salt by washing the treated gluten in DDW, the salt-induced protein-protein interactions are effectively minimized thereby increasing protein-water interactions and ultimately increasing protein solubility. The physical manifestation of a substantially altered protein conformation was evidenced upon washing the 2% NaCl gluten with DDW; it was observed that the residue swelled considerably which was in

marked contrast to the compact viscoelastic residue obtained after DDW extraction of the DDW-prepared gluten. Clements (1973) also found that continued extraction of salt-treated gluten gave a voluminous gel when the salt concentration in the medium was lowered to less than 0.005 M. These results clearly indicate that gluten has been rendered more hydrophilic due to the salt treatment.

Clements (1973) speculated that the increase in hydrophilic properties could result from disruption of gluten complexes involving lipids and polyvalent metal ions. This possibility was investigated by preparing glutens, from chloroform defatted flour, with a solution of EDTA (3 mM) in the absence of salt. No increase in the solubility of gliadin or glutenin was observed. This result indicated that the salt-induced increase of gluten hydrophilicity is not simply due to the disruption of protein-lipid interactions.

The water-extracted proteins from gluten appear to be very sensitive to low levels of NaCl. Addition of NaCl to 1.0 ml of freshly-prepared water-extractable fractions from the 2% NaCl treatment, to make a final NaCl concentration of about 0.3%, precipitated all of the dissolved protein (results not shown). This indicates that a minute quantity of salt is sufficient to neutralize the repulsive forces that maintain the solubility of the gluten proteins in DDW (Bernardin 1978). These results are consistent with those of Preston (1981) who studied the solubility of gluten proteins in solutions of varying concentrations of the lyotropic series of neutral monovalent sodium salts. He concluded that at low salt concentration, the solubility and aggregation of gluten proteins are largely controlled by ionic interactions.

Results presented herein suggest that the relatively low concentration of amino acids

with ionizable side chains in gluten proteins appears to have a significant effect on their solubility in water, and thereby on their rheology in concentrated systems such as doughs. Charged groups which are inaccessible to water molecules (e.g. buried in hydrophobic regions) can generate large electric fields in an environment of low dielectric constant and thereby form strong ion pairs through electrostatic attraction. Once exposed to the outer surface, as induced by salt, the charged groups (as well as additional polar amino acid side chains) become accessible to hydration. Owing to the small net positive charge of gluten proteins at pH 4.8-5.0 (Yoshino and Matsumoto 1966) (the pH of water-soluble fraction 1 from 2% NaCl treated glutens), electrostatic repulsion effects would tend to increase gluten protein solubility in DDW, after removal of the salt. In this condition, even a low concentration of salt can shield the low charge of gluten proteins and consequently lead to aggregation and insolubilization.

Another possible explanation of the observed effect of salt during gluten washing involves the apparent aggregation of gliadin with glutenin during dough formation. In Chapter 3, it was clearly shown that if salt solution is the first solvent used in flour protein fractionation, the subsequent glutenin fraction is substantially contaminated with gliadins, and other monomeric proteins. Dupuis et al (1996) showed that the acetic acid-soluble fraction obtained by the modified Osborne fractionation (where salt solution is the first solvent) contained substantial quantities of gliadin despite the previous extraction with 70% ethanol solution. Thus, the aggregation of gliadin with glutenin appears to be promoted by salt (e.g. gluten washed by salt solution contains more gliadin). It seems plausible therefore that disaggregation of gliadin and glutenin in gluten would likewise

be promoted upon removal of the salt by washing the gluten in DDW, i.e. the gliadin/glutenin complex would be more soluble in DDW than glutenin alone.

Chapter 9

WATER SOLUBILITY OF GLUTENS FROM FLOURS OF DIFFERENT STRENGTH

Abstract

Wet glutens isolated with 0.2% NaCl and 2% NaCl solutions from three Canadian wheat cultivars of diverse dough mixing properties (Glenlea - very strong, Katepwa - strong, Harus - weak) were subjected to four sequential extractions with distilled and deionized water (DDW), producing four water soluble fractions and an insoluble residue. Protein composition of the five fractions was assessed electrophoretically, and the proportions of gliadin and glutenin in each fraction were determined by a selective precipitation procedure. The disaggregation/solubilization behaviour of gluten proteins in DDW showed significant intercultivar differences. For glutens prepared with 0.2% NaCl, essentially all of the gliadin (92.1%) in Glenlea gluten was extracted. The corresponding values for Katepwa and Harus glutens were 76.3%, and 51.0% respectively. Furthermore, the gliadin in the 0.2% NaCl prepared Glenlea gluten was more readily extractable in DDW than gliadin in Katepwa or Harus gluten. The first DDW extraction alone solubilized 57.6% of gliadin in the Glenlea gluten. In contrast, only 4.8% of gliadin in Harus gluten was soluble in the first DDW extraction. Unlike 0.2% NaCl glutens, from which only a small portion of glutenin was extracted by DDW, most of the glutenin in the 2% NaCl glutens was disaggregated and solubilized by DDW extractions. After four sequential DDW extractions, only about 15% and 11% of total

gluten protein remained in the residues of Katepwa and Harus, respectively. The 2% NaCl gluten prepared from Glenlea flour formed a voluminous gel from the beginning of second extraction, which made subsequent DDW extractions impossible. Glutenin in Harus gluten isolated with 2% NaCl was more readily soluble in DDW than that of Katepwa gluten. More than half of total glutenin (54.7%) in 2% NaCl gluten of Harus was solubilized by the first DDW extraction, while the same extraction accounted for only 25.5% of the total glutenin for Katepwa gluten. The implication of these results in relation to the molecular basis of dough mixing properties is discussed.

Introduction

The transformation of hydrated flour particles into a developed dough depends to a large extent on the nature of flour proteins. During dough development, protein aggregates, which at first appear to be particulate or fibrillar in physical structure, are converted into continuous films or membranes that have the right combination of rheological properties for optimum expansion and gas retention during proofing and baking (Bushuk 1985). The process of dough development is generally regarded as encompassing disaggregation/depolymerization of glutenin and interaction of gliadin and glutenin (Mecham 1980; Belitz et al 1986; MacRitchie 1986; Graveland et al 1994; Dupuis et al 1996). During mixing, the glutenin aggregates are hydrated, and apparently unfolded, stretched and associated with themselves and the gliadins. The sulfhydryl-disulfide interchange reactions and non-covalent interactions between gliadin monomers and glutenin polymers are also believed to contribute significantly to the dough mixing process (Bloksma and Bushuk 1988).

Explanation of the intercultivar differences in the dough mixing requirements of wheat flours in molecular terms has posed a considerable challenge for cereal chemists. It has been suggested that the time to peak dough development depends largely on the size, structure, and concentration of glutenin polymers and ratio of gliadin to glutenin (MacRitchie 1986; Graveland et al 1994).

Water is an intermediary for all types of interactions and reactions during dough mixing. The unique breadmaking properties of wheat flour are primarily a function of the behaviour of gluten proteins in an aqueous environment. In Chapter 8, it was

reported that the gluten complex can be virtually disaggregated and dissolved by DDW when the gluten was prepared in the presence of salt. The present study was carried out to compare the disaggregation/solubilization behaviour in water of glutens prepared from wheat cultivars of widely diverse mixing strength. The new information obtained may be useful for further explaining intercultivar differences in dough mixing requirements.

Materials and Methods

Wheat Flour Samples

Flours of Glenlea, Katepwa and Harus, were chosen to represent a wide range of dough mixing properties and breadmaking potential (see Table 4.1 of Chapter 4).

Preparation of Glutens and Their Water-soluble and Residue Fractions

The preparation of wet glutens, and subsequent sequential extraction with distilled and deionized water were carried out according to procedures described in Chapter 8. Gluten was prepared from each flour using two salt (NaCl) concentrations, 0.2% and 2%. The isolated wet glutens, referred to as 0.2% NaCl gluten or 2% NaCl gluten, were subjected to four sequential extractions with DDW, producing four water soluble fractions and an insoluble residue. The glutens and their fractions were freeze-dried. Protein contents (N x 5.7) of the dry preparations were determined by the micro-Kjeldahl method (AACC 1983).

Quantitation of Gliadin and Glutenin in Water-soluble and Residue Fractions

Freeze-dried water-soluble fractions (10 mg) or residue (20 mg) were dispersed in 0.5 ml of 50% 1-propanol. After adding 0.34 ml of 1-propanol to bring the final 1-propanol concentration to 70% (v/v), both 50% 1-propanol soluble and insoluble glutenins were pelleted by centrifugation (15,000*g*, 10 min) (see Chapter 3). The precipitated glutenin was extracted twice (1 h and 30 min, respectively) with 0.75 M NaI (1 ml) to remove the coprecipitated ω-gliadins (see Chapter 5). The gliadins, which are soluble in 70% 1-propanol (after precipitation of glutenin) and in the 0.75 M NaI extract, were thus effectively separated from the glutenin (see Chapter 5). The gliadins in the 70% 1-propanol supernatant and in the 0.75 M NaI extract were combined. Protein contents of the fractions were quantified by micro-Kjeldahl analysis. Because the water soluble fractions of 0.2% NaCl gluten contained only small amounts of glutenin, it was not possible to collect the 70% 1-propanol precipitate for protein quantification. Accordingly, the concentration of glutenin in each of these fractions was calculated by subtracting the sum of protein in 70% 1-propanol supernatant and 0.75 M NaI extract from the protein in the total water-soluble fraction.

Electrophoresis

Subsamples of the original glutens and their fractions were analyzed by acid-polyacrylamide gel electrophoresis (APAGE) according to Sapirstein and Bushuk (1985), and sodium dodecyl sulfate (SDS)-PAGE according to Ng and Bushuk (1987) with the separating gel concentration of 14%.

Results

Solubility of 0.2% NaCl Glutens in DDW

Table 9.1 shows the percentage of gluten protein and their gliadin and glutenin subfractions solubilized in each of four DDW extraction of 0.2% NaCl glutens of the three wheat cultivars. The total percentage of gluten protein extracted by the four DDW extractions was 59.6% for Glenlea, 51.7% for Katepwa, and 32.9% for Harus. The stronger the parent flour from which the gluten was isolated, the greater the proportion of protein that was solubilized.

Results of compositional analysis (Table 9.1) showed that the intercultivar difference was mainly due to the extractability of gliadin in DDW. Essentially all of the gliadin fraction (92.1%) in the gluten from the extra strong Glenlea wheat was extracted. The corresponding values for gliadin of Katepwa glutens were 76.3%, and 51.0%, respectively. It was also noteworthy that gliadin from stronger gluten was more readily extractable in DDW than that of weaker gluten (Table 9.1). The first extraction alone solubilized 57.6% of gliadin in the Glenlea gluten. The equivalent values for Katepwa and Harus were 28.2 and 4.8%, respectively.

The nature of the proteins in the fractions was confirmed by APAGE analysis of the parent 0.2% NaCl glutens, the four DDW soluble fractions, and the residues (Fig. 9.1). It was interesting that only ω-gliadins (the slow-moving components) remained in the DDW insoluble residue from Glenlea gluten (see lane R). In contrast, the gliadin pattern of the residue of Harus gluten was essentially the same as that of unfractionated parent gluten. APAGE patterns also clearly showed the relationship (noted above) between

TABLE 9.1

Protein Solubility (%) in Distilled and Deionized Water of Glutens Prepared with 0.2% NaCl Solution[a]

Gluten Proteins[b]

Cultivar	1st Extract	2nd Extract	3rd Extract	4th Extract	Total Soluble	Residue
Glenlea	38.1±1.2	14.4±0.3	5.0±0.1	2.2±0.3	59.6±1.5	40.4±1.5
Katepwa	19.6±0.8	26.7±0.5	4.2±0.3	1.1±0.1	51.7±0.5	48.3±0.5
Harus	3.2±0.3	17.6±0.2	9.5±1.6	2.6±0.5	32.9±1.1	67.1±1.1

Gliadins[c]

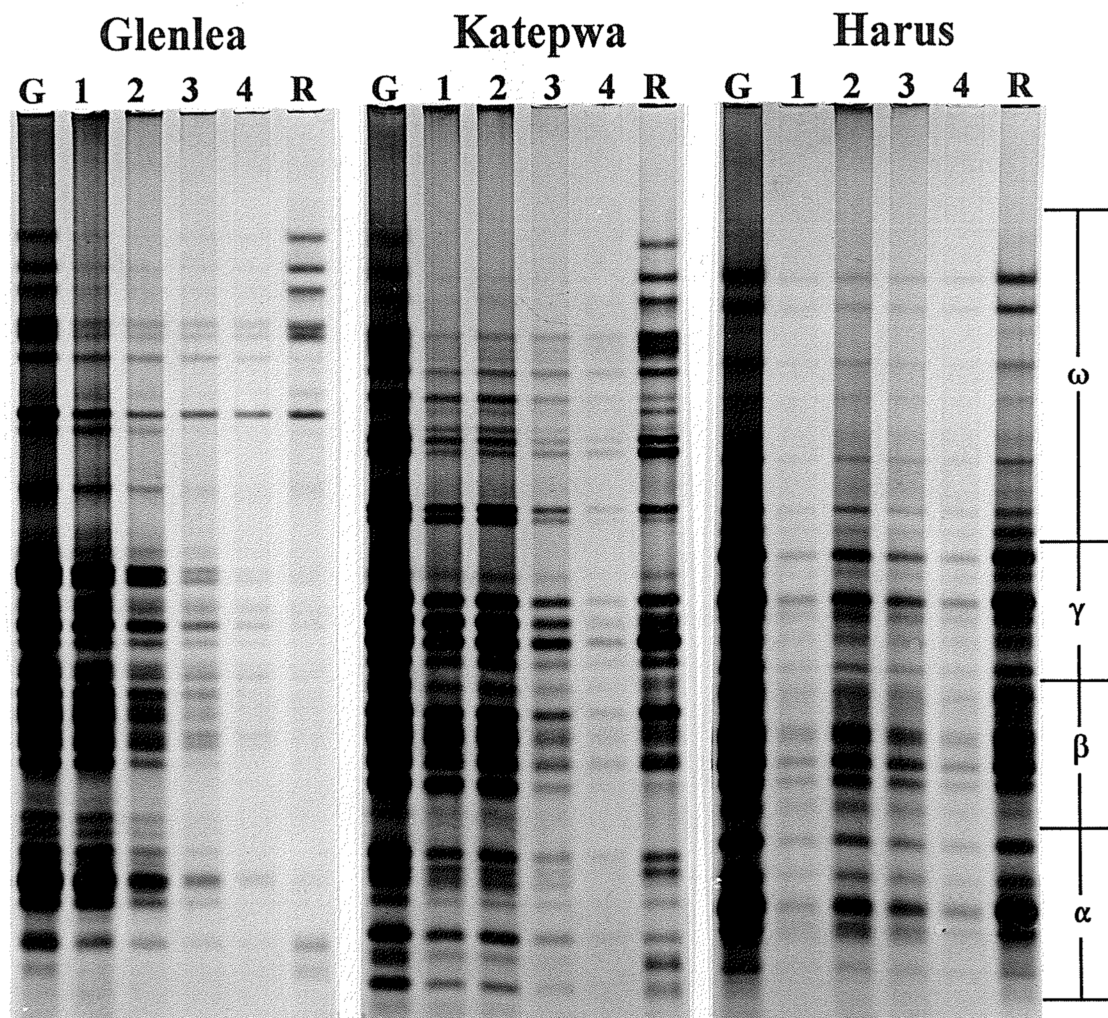
Cultivar	1st Extract	2nd Extract	3rd Extract	4th Extract	Total Soluble	Residue
Glenlea	57.6±1.7	21.9±0.8	8.7±0.6	3.8±0.4	92.1±2.9	7.9±1.0
Katepwa	28.2±1.4	38.9±2.3	7.2±0.8	2.0±0.2	76.3±3.0	23.7±1.0
Harus	4.8±0.6	26.2±1.1	15.6±0.8	4.4±0.0	51.0±1.5	49.0±2.5

Glutenins[d]

Cultivar	1st Extract	2nd Extract	3rd Extract	4th Extract	Total Soluble	Residue
Glenlea	20.2±1.0	7.5±0.6	1.5±0.2	0.6±0.0	29.8±1.4	70.2±1.9
Katepwa	10.7±0.8	13.9±1.2	1.0±0.1	0.2±0.0	25.8±1.0	74.2±1.4
Harus	1.5±0.2	8.3±0.6	2.9±0.2	0.6±0.1	13.3±0.8	86.7±1.7

[a] Means of three replicates ± standard deviation. [b] Percent of sum of the proteins in all fractions.
[c] Percent of sum of the gliadins in all fractions. [d] Percent of sum of the glutenins in all fractions.

Fig. 9.1. Polyacrylamide gel electrophoresis at pH 3.1 of the gliadins of the four sequential water soluble fractions (1-4) and insoluble residue (R) from glutens isolated with 0.2% NaCl solution from Glenlea, Katepwa, and Harus flours. The unfractionated glutens (G) were analyzed as controls.


Glenlea
Katepwa
Harus
G 1 2 3 4 R
G 1 2 3 4 R
G 1 2 3 4 R
ω
γ
β
α

gliadin extractability in the early DDW extractions and dough strength of the samples; the intensity of band staining was in the order Glenlea > Katepwa > > Harus (compare lanes 1 in Fig. 9.1).

In contrast to gliadin solubility in DDW, a much lower amount of glutenin from 0.2% NaCl glutens was solubilized with DDW. It was interesting that the trend observed above in relation to dough strength for gliadin solubility in the first extract was also found for glutenin in 0.2% NaCl gluten; the solubility of glutenin in the first extracts of Glenlea, Katepwa and Harus glutens were 20.2, 10.7 and 1.5%, respectively. The solubility in DDW of this portion of glutenin could be attributed to its strong aggregation with gliadin in the gluten complex. In this case, the amount of glutenin disaggregated in DDW depends on the amount of gliadin solubilized by DDW; more for the stronger cultivars.

Solubility of 2% NaCl Glutens in DDW

As noted in Chapter 8, the 2% NaCl glutens swelled considerably upon sequential extraction with DDW. In fact, the Glenlea gluten formed a very viscous gel from the beginning of the second extraction. Doubling the ratio of solvent to gluten resulted in a voluminous gel from which only about one third of the solvent was recovered after centrifugation. For this reason the 2% NaCl gluten prepared from Glenlea flour was not included in this part of the study.

Although the Katepwa gluten swelled considerably during extraction, only a small amount of viscous gel was obtained after centrifugation of each extraction. The gluten

prepared from Harus also swelled during the early stage of the first extraction, but became fully disaggregated at the end of that extraction, and only a very small quantity of viscous gel remained after centrifugation.

The 2% NaCl glutens of Katepwa and Harus were almost completely disaggregated and dissolved by DDW, leaving only about 15% and 11% of total protein in the residues, respectively, after four sequential DDW extractions (Table 9.2). In this case, essentially all of the gliadin protein and most of the glutenin was extracted with DDW. About 70% of total protein from Harus gluten was solubilized in the first extraction alone; the corresponding value for Katepwa gluten was 39%. Accordingly, the degree and rate of disaggregation in DDW of the 2% NaCl gluten complex appeared to follow the opposite trend compared to that observed for the 0.2% NaCl glutens. Both gliadin and glutenin of Harus gluten were more readily soluble in DDW than that of Katepwa gluten (Table 9.2). The amount of gluten proteins disaggregated and solubilized in DDW appeared to depend on the glutenin component which is considered to be the structural matrix of the gluten complex. More than half of total glutenin (54.7%) in 2% NaCl gluten of Harus was solubilized by the first DDW extraction, while the same extraction accounted for only 25.5% of total glutenin for Katepwa gluten.

SDS-PAGE patterns of the 2% NaCl glutens and their fractions after reduction with 2-mercaptoethanol (Fig. 9.2) clearly showed the presence of HMW subunits of glutenin in all the DDW soluble fractions. Comparison of the pattern of Katepwa and Harus showed that most of the glutenin in Harus gluten was solubilized by the first DDW extraction whereas glutenin in Katepwa gluten was solubilized more slowly.

TABLE 9.2

Protein Solubility (%) in Distilled and Deionized Water of Glutens Prepared with 2% NaCl Solution[a]

Gluten Proteins[b]

Cultivar	1st Extract	2nd Extract	3rd Extract	4th Extract	Total Soluble	Residue
Katepwa	39.0±0.3	32.0±1.9	9.7±0.6	3.9±0.3	84.6±1.6	15.5±1.6
Harus	70.2±1.3	14.1±0.8	3.3±0.2	1.6±0.1	89.2±0.5	10.8±0.5

Gliadins[c]

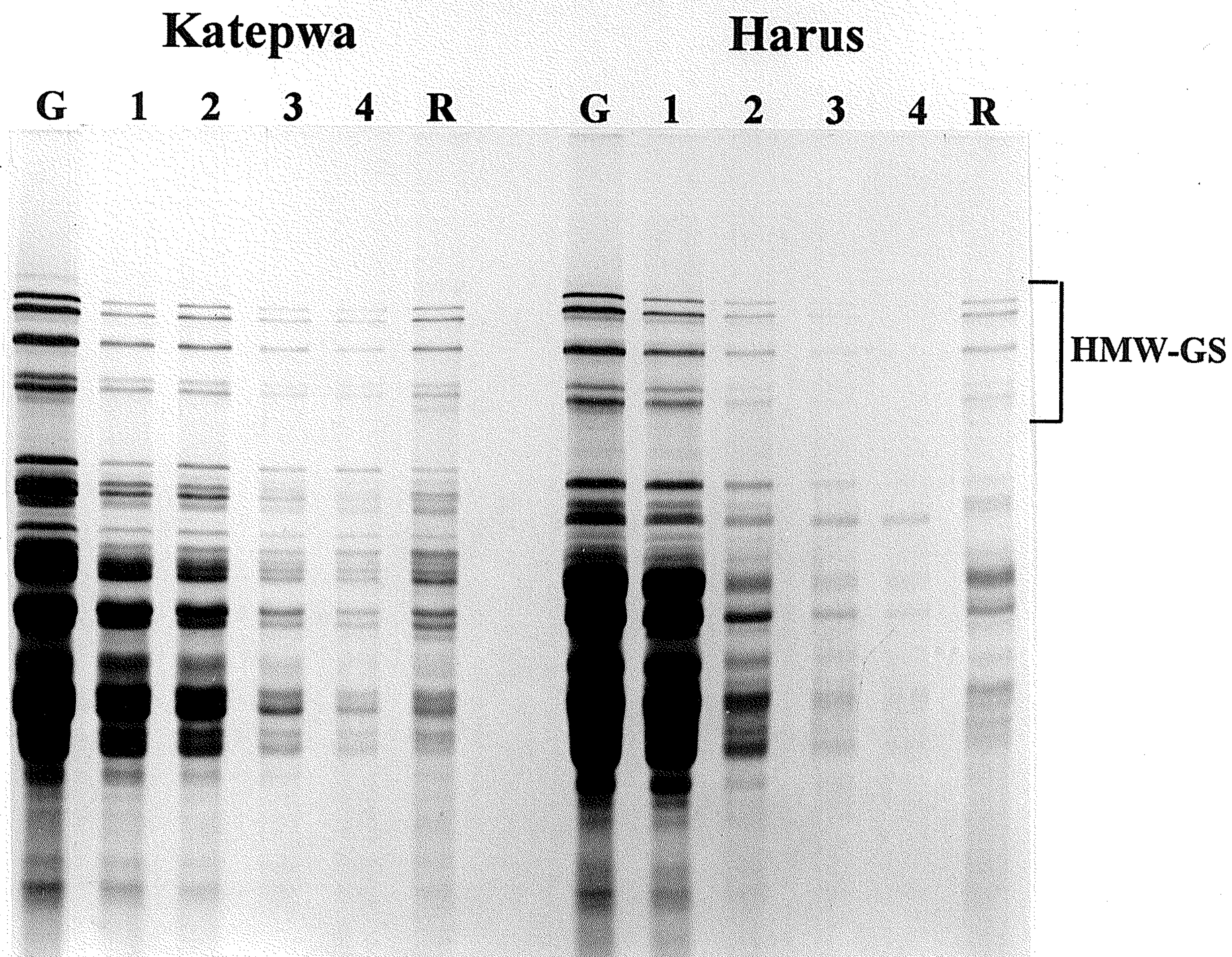
Cultivar	1st Extract	2nd Extract	3rd Extract	4th Extract	Total Soluble	Residue
Katepwa	54.4±1.5	34.3±2.1	7.3±0.5	1.7±0.2	97.6±2.6	2.4±0.3
Harus	86.6±2.5	10.1±0.6	1.6±0.2	0.4±0.0	98.7±2.7	1.3±0.1

Glutenins[d]

Cultivar	1st Extract	2nd Extract	3rd Extract	4th Extract	Total Soluble	Residue
Katepwa	25.5±1.3	30.0±1.9	11.8±0.7	5.8±0.2	73.0±2.1	27.0±0.4
Harus	54.7±2.3	17.9±0.6	4.9±0.2	2.7±0.2	80.2±2.5	19.8±0.3

[a] Means of three replicates ± standard deviation.
[b] Percent of sum of the proteins in all fractions.
[c] Percent of sum of the gliadins in all fractions.
[d] Percent of sum of the glutenins in all fractions.

Fig. 9.2. Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis of reduced four sequential water soluble fractions (1-4) and insoluble residue (R) from glutens isolated with 2% NaCl solution from Katepwa and Harus flours. The unfractionated glutens (G) were analyzed as controls.


Katepwa
Harus
G 1 2 3 4 R
G 1 2 3 4 R
HMW-GS

Discussion

Gluten protein solubility in DDW increased when the gluten was prepared in the presence of salt (see also Chapter 8). The nature and amount of gluten protein solubilized in DDW are related to the salt concentration. DDW extracts of gluten prepared with 2% NaCl contained almost all the gliadin, except some ω-gliadin components, and most of the glutenin. For the gluten prepared with 0.2% NaCl, most of the gliadin, but only a small portion of glutenin, was extracted. The large increase in gliadin and glutenin solubility in DDW when the gluten is prepared in salt solution (after removal of most of the salt) can be explained on the basis of salt-induced conformational change of the proteins, which appear to be more hydrophillic (see Chapter 8).

In addition, this study showed that the disaggregation/solubilization behaviour of gluten proteins in DDW was genotype dependent; the observed intercultivar differences appear to be related to dough mixing properties. Gliadins in strong glutens were more easily extracted by DDW than those of weak glutens. It is generally believed that there exist strong non-covalent interactions, principally hydrogen bonds and hydrophobic interactions between gliadin and glutenin proteins in the gluten complex. In order for the gliadins to be soluble in DDW, the gliadin-glutenin interaction must be replaced by gliadin-water interaction. The degree of this interaction, as reflected by the solubility of gliadins in water, differed significantly between cultivars. It appears that gliadins in weak glutens are more tightly associated with glutenin compared to strong glutens. On the other hand, glutenin in glutens of strong cultivars was more resistant to extraction in DDW than glutenin in glutens of weak cultivars. The precise reason for these differences

is not clear. It has been speculated that the average molecular size of glutenin in strong gluten is larger than that in weak gluten (Gupta et al 1993). The conventional underlying basis for this view is related to the aggregation state of glutenin alone. A different perspective on glutenin molecular size and aggregation has been recently expressed (Sapirstein and Fu 1996). The larger the molecule, the smaller would be the ratio of surface area to mass which determines the degree of inter-molecular protein-protein and protein-solvent interactions. On a constant mass basis, proteins of larger size has lower specific surface areas. Therefore, it seems plausible that gliadin-glutenin interactions in strong glutens would be lower than in weak glutens. It would be easier to solubilize glutenin as a gliadin/glutenin complex than as glutenin alone (Ewart 1980, Huebner and Wall 1980).

The gliadin-glutenin interaction model can be extended to explain the events that occur during dough formation and development. In flour, gluten proteins exist in a dispersed form. On addition of water, the originally closely-packed protein bodies hydrate and begin to break up as the dough is mixed. Dough development, as proposed by Graveland et al (1994), will result from disaggregation/depolymerization of the glutenin component and its further interaction with gliadins. In the process of dough mixing, the gliadins which interact with glutenins act as plasticizers by weakening the mutual interaction between the glutenin aggregates. At the same time, the glutenin becomes more soluble in water by virtue of the fact that the glutenin-gliadin complex is more soluble than the original glutenin. Cultivars with strong gliadin-glutenin interactions (i.e. the gliadin is more resistant to extraction by DDW from flour or gluten complex)

would require less mixing for optimum dough development, and vice versa. This concept is consistent with results of another recent study in our laboratory (Dupuis et al 1996). That study showed that the acetic acid soluble fraction obtained by the modified Osborne fractionation contained substantial quantities of gliadin despite the previous extraction with 70% ethanol solution. The amount of gliadin not extracted by the ethanol and subsequently extracted by acetic acid was inversely related to flour strength. Those results and also this study provide compelling evidence that there is less gliadin-glutenin interaction in flours of greater strength than in weaker flours. The key event that occurs during mixing toward peak development is the conversion of a relatively hydrophobic dough system to one that is more hydrophillic. Water plays a critical role in this change by competing for hydrogen-bonding sites and by effectively solvating interacting polar groups of glutenin moleculars which are progressively decreasing in size during mixing.

The results of the present study showed that there exist significant intercultivar differences in the disaggregation/solubilization rate of glutenin in water. These differences are very likely due to the direct effect of intrinsic differences in the size of glutenin. The indirect effect and inverse relationship proposed between glutenin molecular size and extent of gliadin-glutenin interaction in dough may also be an equally important factor in explaining intercultivar differences in dough mixing requirements.

Chapter 10

GENERAL DISCUSSION

Wheat flour contains two main groups of heterogenous proteins, i.e. polymeric and monomeric, each present in roughly equal quantities in a typical flour (MacRitchie 1992). The term polymeric encompasses proteins consisting of polypeptide subunits linked by disulfide bonds. At a higher level of order likely found only in aqueous environments, disulfide-bonded polymers are aggregated through non-covalent interactions. The polymeric fraction is comprised principally of glutenins (~85%) with small amounts of HMW globulins and albumins. The monomeric proteins are single chain polypeptides and include gliadins (~80%) and albumins and globulins.

Recent research on the biochemical basis of breadmaking quality of wheat flour has intensified the need for an accurate and reliable method for separating polymeric proteins from monomeric proteins. The rationale for such a separation is related to at least four points. First, these two groups of flour protein are functionally distinct, i.e. glutenins and gliadins are associated with dough elasticity and viscosity, respectively. Second, the relative amounts of soluble or insoluble glutenin in a flour appear to be strongly related to the functionality of the flour in breadmaking. Third, after reduction of the disulfide bonds of glutenin, its subunit composition can be used, to a limited extent, to predict the breadmaking potential of a wheat cultivar. Fourth, physicochemical characterization of glutenin requires a pure glutenin fraction free of contaminating monomeric proteins.

The challenges in obtaining a sharp and accurate separation of polymeric and monomeric proteins of wheat flour using differential solubility techniques have been known by cereal chemists for a long time. The well-known overlapping solubilities of gliadin and glutenin proteins combined with the broad solubility characteristics of glutenin itself are the main problems in preparing protein fractions of high purity for analysis and study. In attempting to address the objectives of the thesis research, the first challenge to be met was the discriminative fractionation and quantification of wheat flour protein. This was accomplished using a relatively straightforward procedure based on differential solubility of polymeric and monomeric proteins in different aqueous solutions of 1-propanol.

One of the key features of the fractionation procedure developed was the absence of salt which can cause significant cross-contamination of glutenin with monomeric proteins. Results reported in the scientific literature suggest that principal factors contributing to the cross contamination of some wheat protein fractions are the noncovalent chemical interactions that exist in flour or are induced by the solvents added. Centrifugation of protein-starch dispersions, as in Osborne-type fractionations which begin by water or salt solution extraction of flour, concentrates monomeric and polymeric proteins in the insoluble residues. It seems plausible that under these preparative conditions, noncovalent interactions between monomeric protein and polymeric glutenin would be accentuated. This study has shown that better flour protein fractionation is achievable by initially using 50% 1-propanol to completely remove monomeric proteins.

While it is possible to fractionate monomeric flour proteins and glutenin according

to their differential solubility in 50% 1-propanol, this separation is not complete; a significant quantity of glutenin is also extracted in this solvent. Therefore, a selective precipitation procedure was developed which can separate the 50% 1-propanol soluble (50PS) glutenin from the monomeric protein by precipitation in 70% 1-propanol. The supernatant of this fractionation contains albumins, globulins and most of the gliadin proteins. The selective precipitation of 50PS glutenin also results in co-precipitation of some ω-gliadins. It was found that RP-HPLC is effective in separating the 50PS glutenin from the co-precipitated ω-gliadins, and the amount of glutenin that was soluble in 50% 1-propanol could be quantified. A much simpler method was also developed to selectively remove the contaminating ω-gliadin from the precipitated 50PS glutenin by extraction with 0.75 M NaI without losing any glutenin.

The polymeric proteins in the 50% 1-propanol insoluble residue were further fractionated into two fractions by extraction with 50% 1-propanol containing 1% DTT as a disulfide reducing reagent. Most of the insoluble glutenin was extracted by this solvent. SDS-PAGE and RP-HPLC showed that the 50% 1-propanol/1% DTT soluble fraction consisted of essentially pure HMW- and LMW-GS. However, an appreciable amount of protein remained in the residue even after exhaustive extraction with 50% 1-propanol containing DTT. SDS-PAGE revealed that the final residue contained some HMW-GS and other proteins whose identity was not determined. Interestingly, the HMW-GS were mainly those controlled by *Glu-1D* gene locus. However, the SDS-PAGE results indicated that little if any LMW-GS was present in this final residue.

Four distinct protein fractions were obtained from the fractionation procedure

developed in this study: one monomeric protein fraction and three subfractions of polymeric protein. The latter three correspond to 50% 1-propanol soluble glutenin (precipitated by 70% 1-propanol), 50% 1-propanol insoluble glutenin (soluble after reduction) and residue proteins. In an attempt to relate protein composition to baking quality, the composition of seven wheat cultivars of diverse dough strength was examined by this fractionation procedure and the relationship between the quantity of these fractions and dough properties and breadmaking quality were determined. As expected, the proportion of the variation in physical dough properties and breadmaking quality of the flour samples that was explained by the flour protein fractions were very different.

It is noteworthy that the relative proportion of total monomeric proteins in flour protein was essentially constant (48-52%) among the seven wheat samples. There was no significant relationship between relative proportion of monomeric protein and quality parameters. It has been shown that the viscoelasticity of gluten can be greatly modified by changing the ratio of gliadin to glutenin (Belitz et al 1987; Khatkar et al 1995; Janssen et al 1996). However, this compositional factor is not always related to quality parameters since the size distribution of polymeric protein probably has a larger effect (Gupta et al 1993). Also the intercultivar variation of the ratio of monomeric to total polymeric protein is likely small as found in the present research.

Like the monomeric protein fraction, relatively constant amount of protein, from 14-18% depending on cultivar, remained in the final residue. There was a negative but not significant relationship between the proportion of final residue protein and dough strength parameters (mixograph dough development time and work input to peak, and

extensigraph maximum resistance and area). The effect of the final residue protein on quality is consistent with results obtained by MacRitchie (1987) for the more insoluble fractions of gluten.

The protein fractions with high intercultivar variability were soluble and insoluble glutenins which ranged from 10-20% and 12-28% of flour protein, respectively. These fractions were very closely related to breadmaking quality. In terms of dough strength parameters, the proportions of soluble and insoluble glutenin in flour explained approximately 80-90% of the variation. For loaf volume, the two glutenin fractions accounted for a slightly lower proportion of the variation among samples (70-80%). The parallel influence of both soluble and insoluble glutenin in explaining so much of the variation in breadmaking quality attributes of the sample derives from the very high interdependence of these two protein fractions. This result can be explained by a difference in the average molecular size of the glutenin in the two fractions. It seems reasonable that insoluble glutenin contains a distinctly higher proportion of larger-sized polymers than is present in the soluble glutenin fraction, a view which has been expressed elsewhere (Gupta et al 1993). The strength of the statistical relationship between insoluble glutenin content and quality, combined with the ease with which this glutenin fraction can be isolated from flour and measured (in contrast to soluble glutenin) strongly suggest its utility as a very good biochemical test to discern protein quality differences among different cultivars. Validation of this protein fractionation scheme with other samples including pure cultivars and commercial wheat samples has yielded similar results to those reported in this study (Sapirstein and Suchy 1996).

Results of Chapter 4 showed that glutenins of wheat cultivars with diverse dough strength differ in solubility in 50% 1-propanol. Accordingly, it is essential to know the differences in chemical and physical structures that determine this solubility in order to understand the molecular basis of breadmaking quality. In Chapter 5, the glutenin component of flours of seven Canadian wheat cultivars of diverse dough strength were fractionated into 50% 1-propanol-soluble (50PS) and -insoluble (50PI) fractions. Quantitative and qualitative subunit compositions of the two glutenin fractions were determined by RP-HPLC and SDS-PAGE. Results for each cultivar showed that there was essentially no qualitative difference in subunit composition of the two fractions. Also, there was no difference in the relative amounts of the HMW and LMW subunits determined by RP-HPLC. Results for all seven cultivars showed that 50PI glutenin contained a greater proportion of *Glu-1Dx* subunits, especially *Glu-1Dx5*, and a lesser proportion of *Glu-1Bx* subunits than 50PS glutenin. The proportion of *Glu-1Ax* subunits (i.e. *Glu-1Ax1* and *Glu-1Ax2**), *Glu-1By* subunits (i.e. *Glu-1By8* and *Glu-1By9*) and *Glu-1Dy* subunits (i.e. *Glu-1Dy10* and *Glu-1Dy12*) was essentially constant. For two of the seven cultivars (Glenlea and AC Karma), the proportion of a D group LMW subunit (D*) of the total LMW subunits in 50PS glutenin was more than two times the proportion in 50PI glutenin. The results obtained in this study indicate that *Glu-1Dx* subunits are most important (at least for the seven genotypes examined) to the formation of the less soluble glutenin (larger molecular size) which, quantitatively, appears to be directly related to dough strength. This finding could be helpful in devising strategies for breeding wheat cultivars with improved breadmaking quality.

The correlations obtained in Chapter 4 give clues about the association of quality and composition, but high correlations do not always guarantee that a cause and effect relationship exists. Fractionation and reconstitution/fortification procedures provide opportunity to test directly whether specific fractions exert the effects on dough properties and breadmaking quality suggested by correlations. An important consideration in reconstitution experiments is that there is no change in functionality of flour constituents, a requirement not easily attained in view of the labile nature of flour proteins. Another major problem that often occurs in reconstitution studies is the high cross contamination between gliadin and glutenin fractions, and differences in the composition of the fractions obtained by various procedures. The heterogeneity of the fractions prepared in some reconstitution studies questions the use of terms gliadin and glutenin to describe them (Chakraborty and Khan 1988a).

Chapter 6 presented new information on the subject of fractionation and fortification based on an experimental approach that is substantially different from that used by other authors (Hoseney et al 1969a, 1969b; Goforth et al 1977; MacRitchie 1978, 1985, 1987; Booth and Melvin 1979; Preston and Tipples 1980). Gliadin-rich and glutenin-rich fractions were isolated from 0.5 M NaCl insoluble residues of three Canadian wheat flours by repeated extraction with water. The three flours differed widely in breadmaking potential. The solvent (water) and physical treatment (mild magnetic stirring), used for preparation of the two fractions were very mild compared to methods based on dissociating solvents and/or high shear homogenization used by some workers. Electrophoretic and compositional analysis showed that the separation of gliadin and

glutenin was very effective. Upon addition to base flours and analysis by the 2 g computerized mixograph, the gliadin-rich and glutenin-rich fractions caused significant weakening and strengthening of dough, respectively. In regard to cultivar effects, the gliadin fraction of the three cultivars with very different dough strengths (as well as gliadin composition) gave essentially identical dough-weakening effect at corresponding levels of protein enrichment. In contrast, the degree of dough-strengthening effects of the glutenin-rich fractions was cultivar dependent, i.e. it was directly related to the strength of the original flour from which the fraction was isolated. The glutenin-rich fraction isolated from Glenlea produced the greatest increase in dough strength of the very weak base flour. This dough-strengthening effect was lower for Katepwa glutenin, and lower still for SWS-52 glutenin, the weakest of the three flours.

These protein enrichment results confirmed those of Chapter 4 that were based on statistical correlations. Together these results showed that the quality differentiating factor of gluten proteins resides in the glutenin fraction. Quantitative variation of gliadin is very small among the wheat genotypes studied. Qualitative variation of gliadin, albeit very extensive and useful in wheat cultivar identification, does not appear to contribute to the observed intercultivar differences in mixing properties. In contrast, the effect of the glutenin fraction was cultivar dependent and appears to wholly account for the intercultivar differences in flour strength.

Glutenins are comprised of two sets of relatively distinct polypeptides (HMW and LMW subunits), bonded together by disulfide bridges into very large molecules. Once the key role of glutenin protein in governing cultivar differences in breadmaking potential

was established, the next step was to study the separate effects of HMW- and LMW-GS.

Much of the research during the last 15 years on the molecular basis of breadmaking potential has focused on the variation and genetic control of glutenin subunits and their association with wheat end-use quality (Payne 1987; Shewry et al 1992; Gupta et al 1995). Excellent progress has been made on the structure/functionality of the subunits of glutenin. Currently available evidence indicates that both qualitative and quantitative differences in HMW and LMW subunits appear to contribute to intercultivar variation in breadmaking quality of wheat flour. However, understanding of the functionality of glutenin subunits has been mainly based on correlative studies, which are valid for identifying marker proteins but do not necessarily indicate cause-and effect relationships. Recently, a technique for incorporating subunits into wheat glutenin by partial reduction, followed by oxidation has been developed by Bekes et al (1994a). This method can be used to directly study the functionality of specific glutenin subunit(s). However, interpretation of results should be approached with caution as "incorporation" of subunits by chemical polymerization no doubt results in an overall glutenin structure that is probably very different from that which exists in nature.

In Chapter 7, HMW- and LMW-GS fractions were isolated from three different wheat cultivars of diverse dough strength. The HMW- and LMW-GS were chemically incorporated separately into doughs of a base flour, and their effects on the mixing properties of the doughs were evaluated with a 2 g computerized mixograph. The results indicated that incorporation of LMW-GS from different wheat cultivars had relatively marginal effects on the dough mixing properties of the base flour. In contrast,

incorporation of HMW-GS resulted in a substantially significant increase in dough strength, however, no qualitative intercultivar differences in terms of dough-strengthening effects were observed among the HMW subunit isolates.

Using genetic lines lacking one, two or all three *Glu-3* loci, Gupta et al (1995) showed that LMW subunits contributed positively to dough strength, although far less than the HMW subunits. Many studies have shown the relationship between allelic variation in the polypeptide composition of the HMW-GS and technological properties of wheat (for review see Payne 1987, and MacRitchie et al 1990). Neither the significant influence of differences in HMW-GS composition, nor the positive effect of LMW-GS on dough strength could be established by the chemical incorporation experiments that were performed. Results of incorporation (Chapter 7) seem to be particularly at odds with results obtained by fortification of glutenin-rich fraction (Chapter 6) which gave clear intercultivar differences in mixing characteristics. The reason for the variance is not clear. A possible explanation is that in order to isolate total HMW-GS (and LMW-GS) for chemical incorporation, the intrinsic structure of polymer (i.e. molecular size) must be destroyed, along with any qualitative differences which may have existed among different glutenins.

Water is an intermediary for all types of interactions and reactions during dough formation. Accordingly, it is important to know the physicochemical behaviour of gluten proteins in water if the molecular basis of their unique functionality in dough is to be fully understood. In preliminary experiments related to this thesis research, a large increase in the solubility of gliadin and glutenin in water was observed when the parent

gluten was prepared in the presence of salt. Experiments described in Chapter 8 were undertaken to explore this unusual phenomenon in more detail and to examine the effect of salt concentration used in preparing gluten on the subsequent dissolution of gluten in water. It was found that gluten protein solubility increased with increasing salt concentration. The DDW solubility of Katepwa gluten proteins prepared in DDW, 0.2% NaCl and 2% NaCl were 27, 52, and 85%, respectively after four sequential extractions with DDW. The large increase in the solubility obtained in this experiment was explained on the basis of a salt-induced conformational change of the proteins which renders them more soluble in water. The physical manifestation of a substantially altered protein conformation was evidenced during extraction of the 2% NaCl gluten with DDW; it was observed that the residue swelled considerably which was in marked contrast to the compact viscoelastic residue obtained after DDW extraction of the DDW-prepared gluten. It appears that the salt treatment rendered the gluten proteins more hydrophillic. Two hypotheses are proposed to explain this result, 1) ionic effects and 2) gliadin-glutenin interactions.

For the hypothesis concerning ionic effects, these results suggest that the relatively low concentration of amino acids with ionizable side chains in gluten proteins appears to have a significant effect on their solubility in water, and thereby on their rheology in concentrated systems such as doughs. Charged groups which are inaccessible to water molecules (e.g., buried in hydrophobic regions) can generate large electric fields in an environment of low dielectric constant and thereby form strong ion pairs through electrostatic attraction. Once exposed to the outer surface, as induced by salt, the charged

groups (as well as additional polar amino acid side chains) become accessible to hydration. It is postulated that salt has two effects on gluten proteins. It induces a conformational change which exposes charged groups on the surface of the protein molecules/aggregates; this would lead to repulsion, hence increased solubility in DDW, after the salt is removed. Secondly, even low concentration of salt can shield the low charge of gluten proteins and thereby indirectly promote formation of hydrogen bonds and hydrophobic interactions leading to aggregation and insolubilization. The well-known dough strengthening effect of NaCl can be explained by the charge shielding effect.

An alternative explanation for the increased hydrophillicity of gluten proteins due to salt treatment involves interactions between gliadin and glutenin. Results of Chapter 9 showed that the disaggregation/solubilization behaviour of gluten proteins in DDW was genotype dependent; the observed intercultivar differences appear to be related to dough mixing properties. Gliadins in strong glutens were more easily extracted by DDW than those of weak glutens. It is generally believed that there exist strong non-covalent interactions, principally hydrogen bonds and hydrophobic interactions between gliadin and glutenin proteins in the gluten complex. For the gliadins to be soluble in DDW, the gliadin-glutenin interaction must be replaced by a gliadin-water interaction. The degree of this interaction, as reflected by the solubility of gliadins in water, differed significantly between cultivars. It appears that gliadins in weak glutens are more tightly associated with glutenin compared to strong glutens. On the other hand, glutenin in glutens of strong cultivars was more resistant to extraction in DDW than glutenin in glutens of weak cultivars. The precise reason for these differences is not clear. It has been

speculated that the average molecular size of glutenin in strong gluten is larger than that in weak gluten (Gupta et al 1993). The larger the molecule, the smaller would be the ratio of surface area to mass which determines the degree of inter-molecular protein-protein and protein-solvent interactions (Sapirstein and Fu 1996). On a constant mass basis, proteins of larger size has lower specific surface areas. Therefore, it seems plausible that gliadin-glutenin interactions in strong glutens would be lower than in weak glutens. Because of the higher hydrophilicity of gliadin compared with that of glutenin, it should be easier to solubilize glutenin as a gliadin/glutenin complex than as glutenin alone.

The gliadin-glutenin interaction hypothesis can be extended to explain the events that occur during dough formation and development. In flour, gluten proteins exist in a dispersed form. On addition of water, the originally closely-packed protein bodies hydrate and begin to break up as the dough is mixed. Dough development, as proposed by Graveland et al (1994), will result from disaggregation/depolymerization of the glutenin component and its further interaction with gliadins. In the process of dough mixing, the gliadins which interact with glutenins act as plasticizer by weakening the mutual interaction between the glutenin aggregates. At the same time, the glutenin becomes more soluble in water by virtue of the fact that the glutenin-gliadin complex is more soluble than the original glutenin. Cultivars with strong gliadin-glutenin interactions (i.e. the gliadin is more resistant to extraction by DDW from flour or gluten complex) would require less mixing for optimum dough development, and vice versa. This concept is consistent with results of another recent study in our laboratory (Dupuis et al 1996).

That study showed that the acetic acid soluble fraction obtained by the modified Osborne fractionation contained substantial quantities of gliadin despite the previous extraction with 70% ethanol solution. The amount of gliadin not extracted by the ethanol and subsequently extracted by acetic acid was inversely related to flour strength. Those results and also this study provide compelling evidence that there is less gliadin-glutenin interaction in flours of greater strength than in weaker flours. The key event that occurs during mixing toward peak development is the conversion of a relatively hydrophobic dough system to one that is more hydrophillic. Water plays a critical role in this change by competing for hydrogen-bonding sites and by effectively solvating interacting polar groups of glutenin molecules. The results of Chapter 9 showed that there exists significant intercultivar differences in the disaggregation rate of glutenin in water.

In conclusion, substantial evidence has been offered that intercultivar differences in breadmaking quality, principally physical dough properties, derives from quantitative and qualitative differences in polymeric glutenin, i.e. insoluble glutenin concentration and presumably molecular size, respectively. That HMW-GS is the key component of glutenin also seems clear. These quantitative and qualitative factors can explain protein solubility phenomena, protein-protein interactions and a large part of dough rheological behaviour. In this regard, the flour sample with the highest ratio of insoluble to soluble glutenin, as well as marginally the lowest concentration of monomeric proteins was Glenlea which possesses very strong dough mixing characteristics. These compositional results provide new evidence to explain the biochemical nature of the extra strong dough properties of Glenlea wheat.

Chapter 11

CONTRIBUTIONS TO KNOWLEDGE

1. A new fractionation procedure was developed for separation of monomeric and polymeric proteins of wheat flour based on their differential solubility in different aqueous solutions of 1-propanol. Pure glutenin can be isolated from gliadin-glutenin mixtures using this procedure.

2. A residue protein fraction was identified which is insoluble in 50% 1-propanol containing reductant. The nature of residue protein insolubility was not due to the possibility that the protein was not in a reduced form; subunits were in fact reduced but not extractable in 50% 1-propanol. This polymeric protein fraction represented approximately 16% of total flour protein and was comprised mainly of *Glu-1D* glutenin subunits and non-prolamin polypeptides.

3. The relative amount of monomeric proteins in total flour protein was essentially constant (~50%) among wheat cultivars of very different quality. The protein fractions with significant intercultivar variability were 50% 1-propanol-soluble (50PS) (10-20% of total flour proteins) and -insoluble (50PI) glutenins (12-28% of total flour proteins). The proportions of 50PS and 50PI glutenins were very closely related to physical dough properties, particularly mixing times and work input to optimum dough development.

4. There was essentially no qualitative difference in subunit composition of the 50PS and 50PI glutenin fractions. Also, there was no difference in the relative amount of the high molecular weight (HMW) and low molecular weight (LMW) subunits in the two fractions determined by RP-HPLC.

5. Significant quantitative differences existed in the subunit compositions of 50PS and 50PI glutenins. 50PI glutenin contained a greater proportion of *Glu-1Dx* subunits, especially *Glu-1Dx*5, and a lesser proportion of *Glu-1Bx* subunits than 50PS glutenin. The *Glu-1Dx* subunits appear to be most important to the formation of more insoluble glutenin (50PI) whose concentration was directly related to dough strength.

6. A "gentle" fractionation procedure was developed to prepare relatively pure and functional gliadin and glutenin fractions for reconstitution studies. This fractionation procedure avoids the use of denaturing solvents and/or high-shear mixing.

7. Based on protein reconstitution/enrichment experiments, qualitative variations in gliadin proteins do not contribute to intercultivar differences in dough mixing properties. In contrast, a qualitative factor in glutenin (presumably molecular size) is a major determinant of differences in dough mixing properties among wheat cultivars.

8. Chemical incorporation into doughs of total LMW-glutenin subunits isolated from different cultivars had relatively marginal effects on the dough mixing properties of a base flour. In contrast, chemical incorporation of full complements of HMW-glutenin subunits resulted in a significant increase in dough strength, however no qualitative intercultivar differences in terms of dough-strengthening effects was observed among the HMW subunit isolates.

9. The solubility in water of gliadin and glutenin proteins can be increased substantially when the gluten is prepared in salt solution (after removal of the salt); the higher the salt concentration, the higher the solubility.

10. The disaggregation/solubilization behaviour of gluten proteins in water is genotype dependent and related to dough mixing properties. Gliadins in gluten of stronger mixing wheats were more easily extracted by water than gliadins in gluten of weak wheats.

11. In light of experimental results, a new hypothesis was developed concerning gliadin-glutenin interactions in dough. The hypothesis basically states that the strength of interaction (mediated by the molecular size distribution of glutenin) is inversely related to dough mixing requirements.

Chapter 12

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Appendix: Technological Characterization of the Seven Wheat Cultivars

Seven wheat cultivars were chosen to represent a wide range of dough properties and baking potential. Glenlea, Roblin and Katepwa are hard red spring wheat cultivars, with extra strong, very strong, and strong dough characteristics, respectively. Roblin and Katepwa are wheats belonging to the Canada Western Red Spring class, while Glenlea is a wheat cultivar of the Canada Western Extra Strong class. Biggar and AC Karma are cultivars of the Canada Prairie Spring wheat class. They exhibited strong and moderately strong dough properties, respectively. SWS-52 and Harus are soft white spring and soft white winter wheats, respectively. Both have very weak dough properties. Falling Number values (AACC method 56-81B, 1983) were measured for wholemeal samples of the seven cultivars to ensure that the wheat was sound. The homogeneity of each sample was verified by both PAGE (Sapirstein and Bushuk 1985) and SDS-PAGE (Ng and Bushuk 1987).

Glenlea, Roblin, Katepwa, Biggar and AC-Karma were milled to straight grade flour on a Buhler pneumatic laboratory mill. SWS-52 and Harus were similarly milled on an Allis Chalmers laboratory mill. Dough mixing properties were measured using a 2-g direct drive computerized Mixograph (National Manufacturing, Lincoln, Nebraska). Extensigraph data were obtained according to AACC method 54-10 (AACC, 1983). The remix-to-peak baking test (Kilborne and Tipples, 1981) was used to evaluate the baking quality of the flours. This test evaluates baking potential of flour using optimized conditions of water absorption and dough development.

Quality Characteristics of Flour Samples[a]

Cultivar[b]	Wholemeal FN (sec)	Flour Yield (%)	Flour Ash[c] (%)	Flour Protein[c] (%)	Farinograph FAB (%)	Farinograph DDT (min)	Mixograph DDT (min)	Mixograph WIP (%Tq*min)	Extensigraph Rmax (BU)	Extensigraph Ext (cm)	Extensigraph Area (cm^2)	Loaf Volume (ml)
Glenlea	476	74.3	0.42	13.7	61.8	22.0	5.3	211	1000	19.0	296	828
Roblin	413	73.7	0.41	13.6	63.2	11.5	3.4	141	560	23.0	196	1010
Katepwa	481	73.4	0.45	13.3	64.8	9.0	3.5	123	590	16.7	146	913
Biggar	434	71.8	0.40	12.5	56.7	14.0	3.7	141	720	22.5	134	970
AC Karma	478	76.5	0.46	10.9	56.3	4.0	2.6	69	390	18.0	108	603
SWS 52	325	73.6	0.46	9.5	54.6	2.0	1.9	42	245	17.5	61	423
Harus	295	75.3	0.44	9.5	52.2	1.2	2.0	30	170	16.0	41	355

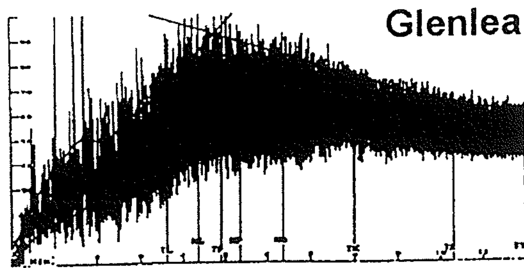
[a] means of at least duplicates

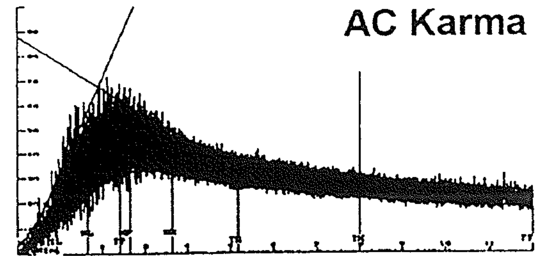
[b] ranking based on wheat class and mixing strength within class

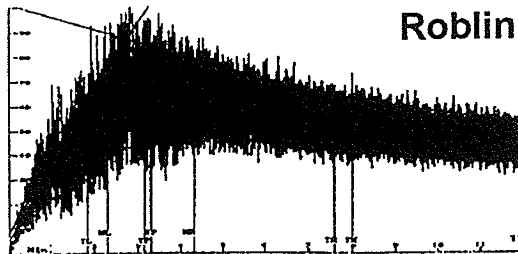
[c] 14% m.b.

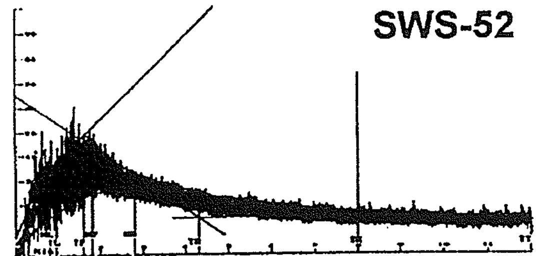
Abbreviations used: FAB = farinograph water absorption; DDT = dough development time; WIP = work input to peak; Rmax = maximum resistance; Ext = extensibility.

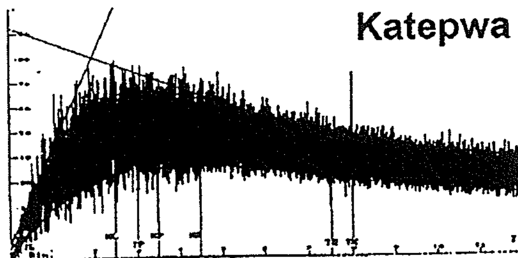
Mixograph Curves

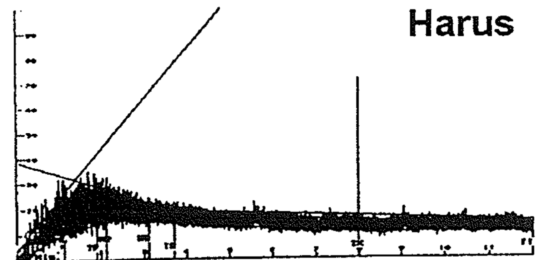


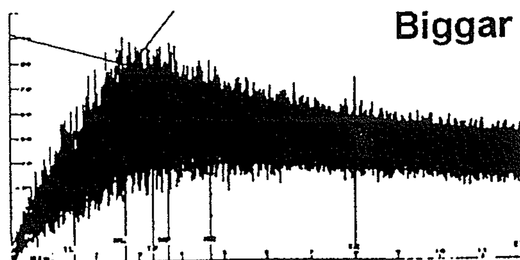




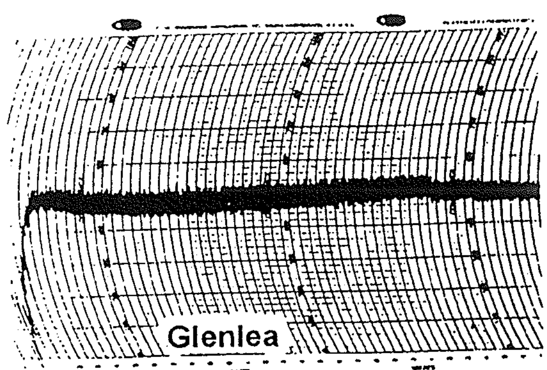


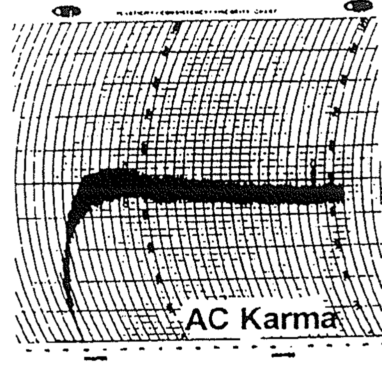


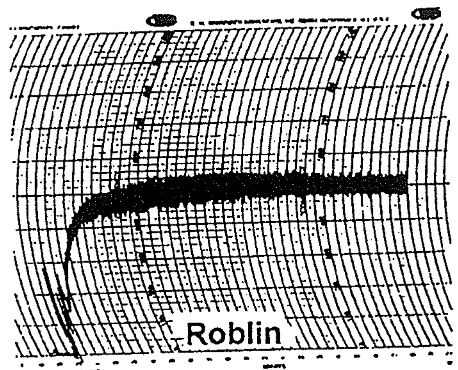


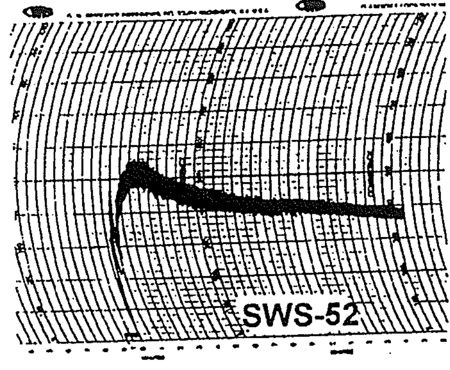


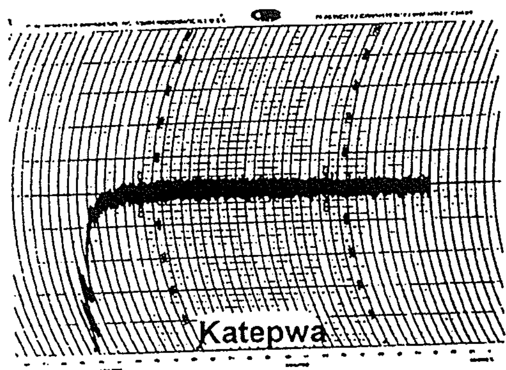
Farinograph Curves

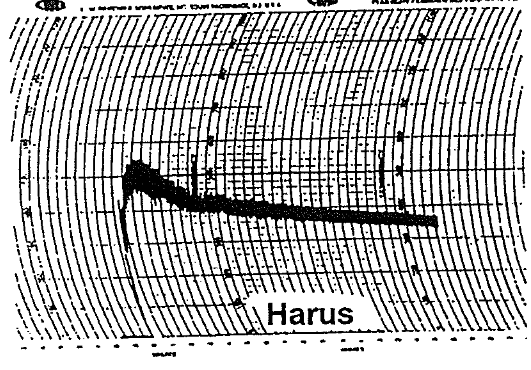


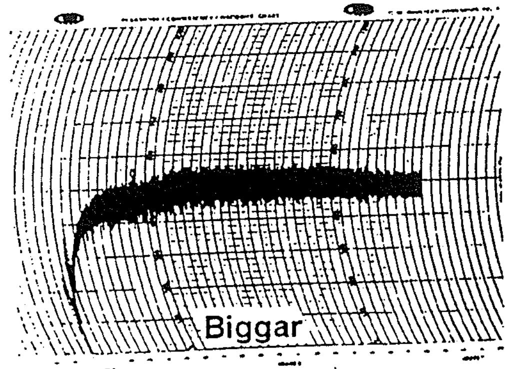












Extensigraph Curves