Examination of the Structure of the Glutenin Macropolymer in Wheat Flour and Doughs by Stepwise Reduction

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The structure of the disulfide-bonded glutenin macropolymer (GMP) in flour and dough was studied by analyzing the depolymerization behavior during stepwise chemical reduction. During reduction, the size distribution of GMP changed, due to the release of subunits, dimers, and oligomers as well as small glutenin aggregates. Glutenin subunits were released from the polymer in a nonrandom order, which was indicative of the polymer having a hierarchical structural organization. Typically, the B-mobility low molecular weight glutenin subunits (LMW-GS) were released at lower reductant concentration than C-mobility LMW-GS and high molecular weight glutenin subunits (HMW-GS), although the B-subunits were released in a partially reduced state. Dimers comprised of either LMW- or HMW-GS were released at low reductant concentrations, but LMW- and HMW-GS were not found together in the same dimers. Some HMW-GS were particularly resistant to reductive dissociation, consistent with them forming a backbone to the glutenin macropolymer. Antibodies specific for different LMW-GS N-terminal sequence families were used to demonstrate that different families were released at different reductant concentrations. Only some of the sequence families were associated with LMW dimers. Individual subunits and dimers more readily depolymerized from overmixed doughs compared with flours. These studies suggest that the glutenin macropolymer in flours has a well-ordered structure that can be modified by dough mixing.

Keywords: *Flour; dough; protein; glutenin; disulfide; reduction*

INTRODUCTION

The glutenin macropolymer in wheat flour is composed of high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits which are characterized based on their genetic derivation, size, and specific sequence characteristics (Payne and Corfield, 1979). Bread wheat is an allohexaploid containing three genomes and seven pairs of chromosomes. Glutenin proteins are comprised of high molecular weight glutenin subunits (HMW-GS) which are encoded by single loci located on the long arms of chromosome 1A, 1B, and 1D and low molecular weight glutenin subunits (LMW-GS) which are encoded for primarily on the short arms of chromosome 1A, 1B, and 1D (Payne, 1987 and Skerritt and Tatham, 1996). HMW-GS are subdivided into x- and y-type subunits based on differences in amino acid composition and structure (Payne et al., 1981 and Shewry et al., 1989), and LMW-GS into B- and C-type subunits based on their mobility on SDS−PAGE. The precise subunit composition of a given flour relates to the quality of the flour and consequently end use suitability (Khelifi and Branlard, 1992). The subunit molecular weight ranges from 36 000 to 44 000 for LMW-GS and approximately 60 000−90 000 for HMW-GS, but as a polymer, the molecular mass may be in excess of 100 000 000 (Shewry et al., 1992). These subunits are associated with each other through disulfide bonding, forming the glutenin macropolymer which is responsible for the viscoelastic properties characteristic of a dough. The relationship between structure and function is poorly understood, and to date much of the understanding of the structure of the GMP is based on the primary (sequence type) and to some extent the secondary structure of individual subunits.

Understanding the structure of the GMP is important for an increased appreciation of the structure−function relationship. Although the ratio between glutenins and gliadins in a flour affects its viscoelastic properties, the glutenin fraction appears to be the major determinant of quality between cultivars (Khatkar et al., 1995). Previous researchers have utilized chemical depolymerization techniques to study the structure of the GMP; however, their work has focused on determining the composition of oligomers and dimers released from the GMP and examination of the release characteristics of HMW-GS (Matsumura et al., 1984; Kawamura et al., 1985). Analysis of the composition of dimers of HMW-GS indicated that x−y dimers exist as well as x−x dimers, but there was no evidence supporting the presence of y−y dimers. The likely sites of intermolecular bonding of HMW-GS dimers were proposed (Werner et al., 1992). In addition to studying the structure of the GMP directly using chemical reduction, the functional properties of normal and of heat-treated glutenins have been examined (Lavelli et al., 1996). Dough treated with reducing agent has markedly different mixing characteristics than untreated dough (Ng et al., 1991; Gao et al., 1992). Differences in the extractability of glutenin proteins using the reducing agent dithiothreitol have been postulated to be due to differences in hydrophobicity (Kim and Bushuk, 1995).

The aim of the current research was to understand the role of both the LMW- and HMW-GS in the structural organization of the GMP using stepwise reduction. The order of subunit release with respect to

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release of high and low molecular weight subunits was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis and densitometry. Included is a comparison of the release patterns of B- and C-LMW-GS, specific N-terminal sequence types, and dimers composed of LMW-GS or HMW-GS. In particular, the role of different HMW-GS in flours was studied using genetic lines lacking particular chromosomes and thus lacking the glutenin subunits encoded by those chromosomes. The composition of oligomers and dimers released at low reductant concentrations was examined using both two-dimensional SDS−PAGE and immunoblotting with a panel of antibodies that were highly specific for individual N-terminal sequence families.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT) was obtained from Diagnostic Chemicals Ltd., Prince Edward Island, Canada. 4-Vinylpyridine (4-VP) was obtained from Sigma (St. Louis, MO). All solvents were analytical grade except those used for high performance liquid chromatography (HPLC) (Mallinckrodt, Paris, KY). Acrylamide and *N*,*N*-methylbisacrylamide were purchased from BioRad Laboratories (Hercules, CA). *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine was purchased from Merck and ammonium persulfate from Boehringer Mannheim (Indianapolis, IN). All buffers were made from reagent grade chemicals using MilliQ water (Millipore, Bedford, MA).

Flours. Chinese Spring variety flour (glutenin subunit composition: HMW-GS: null, 7+8, 2+12, LMW-GS: a, a, a) and three Chinese Spring genetic lines lacking individual group 1 chromosomes (nulli1A-tetra1B, nulli1B-tetra1A, nulli1D-tetra1A) were used for this study in order to examine the chromosomal influence on release of subunits from the GMP. Dough was prepared from a flour with the glutenin subunit composition (HMW-GS: b, i, a [2*, 17+18, 2+12]; LMW-GS: b, b, b) of pedigree RAC5204;MND/4*RAC177/5/RAC565; MILLEWA/4/VCM/CNO'S′//CAL/3/ NORQUAY, and mixed in a 2 g Mixograph using a modification of the standard method for 35 g flour scaled to 2 g size (Gras and O'Brien, 1992). In this study, the LMW-GS alleles are described using the letter coding system developed by Singh and Shepherd (1988) and identified using the SDS−PAGE system of Singh et al. (1991).

Sample Extraction. Samples for protein assay (using Coomassie G-250 dye binding [Bradford, 1976]), size-exclusion HPLC, and SDS−PAGE were prepared as follows. Glutenin from 60 mg of flour or dough was prepared by first removing other proteins by four pre-extractions in 1 mL of 50% (v/v) *n*-propanol, with vortex mixing then constant shaking at room temperature. Each extraction took place over 30 min, followed by centrifugation in a microcentrifuge at 13 160 *g* for 10 min. The pellet was partially reduced by shaking for 16 h with 50% (v/v) *n*-propanol−125 mM Tris-HCl, pH 7.5, containing one of a range of concentrations of dithiothreitol. The DTT concentration range used was 0, 0.1, 0.3, 0.5, 0.7, 1, 2, 3, 4, 5, 20, 50, and 100 mM. A "fully reduced" sample was also prepared by extraction in 65 mM (1%, w/v) DTT followed by complete reduction with 286 mM [2% (v/v)] 2-mercaptoethanol (modified from Singh et al., 1991). For electrophoresis, polypeptides were alkylated by adding 4-VP to 86.7 mM [0.93% (v/v)] final concentration. Preliminary experiments demonstrated that this treatment did not alter the polypeptide profile using one-dimensional SDS−PAGE but sharpened many of the bands. Samples analyzed by SE-HPLC were not alkylated since some of the unreacted 4-VP in the sample coeluted with some of the lower molecular weight polypeptides.

Electrophoresis. Flour extracts were mixed with an equal volume of sample buffer concentrate (250 mM Tris-HCl, pH 7.5, 4% (w/v) SDS, 20% (v/v) glycerol). Samples were fractionated on 12% T, 2% C polyacrylamide gels, run at constant voltage for 1800 Vh. Stacking gels were 6% T acrylamide and 2% T. For two-dimensional (partly reduced × fully reduced) SDS−PAGE, the first dimension gel was either 8% T, 2% C or 12% T, 2% C, run for 1800 Vh, and then cut into 1 cm-wide strips. After equilibration for 2 × 1 h at room temperature in 125 mM Tris-HCl, pH 7.5, 2% (w/v) SDS, 10% (v/v) glycerol, containing 4% (v/v) 2-mercaptoethanol, the strip was placed horizontally across the sample well of a second 12% T, 2% C gel and run for further 1800 Vh. A sample of fully reduced GMP was run in a neighboring lane in each gel. All gels were 1.5 mm thick except for the first dimension of two-dimensional gels, which was 1 mm thick. Gels were either stained in high sensitivity colloidal Coomassie G-250 (BioRad) in methanol-ammonium sulfate and phosphoric acid (Neuhoff et al., 1991) or blotted on polyvinyl difluoride (PVDF) membrane (Micron Separations Inc., Westborough, MA). Broad range molecular weight markers (Biorad) were used in order to characterize the molecular weight of dimers and oligomers. The molecular weight standards included myosin (200 000), β-galactosidase (116 250), phosphorylase B (97 400), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), lysozyme (14 400), and aprotinin (6500).

Densitometry. Stained gels were scanned densitometrically using an LKB Ultroscan XL Laser Densitometer using Gelscan XL software (LKB, Bromma, Sweden), with settings as follows: one-dimensional analysis, smoothing: 2, peak width: 1, peak number: 35, peak area: 0.1, baseline: 1, x-width: 1.

Semidry Electroblotting and Antibody Probing. Polypeptides fractionated on SDS−PAGE gels were transferred to polyvinylidine difluoride (PVDF) membranes as follows. Prior to blotting, SDS−PAGE gels were equilibrated for 30 min in transfer buffer (25 mM Tris and 192 mM glycine and 0.02% (w/v) SDS and 10% methanol). Transfer occurred over 6 h at 250 mA, using 40 mM 6-aminocaproic acid and methanol (20%), buffered with Tris to pH 7.5, as the cathode buffer, and 25 mM Tris and 300 mM Tris in 20% methanol as the inner and outer anode buffers, respectively. PVDF membrane was wet in methanol prior to blotting. After blotting, PVDF membranes were fixed for 5 min in 1% (w/v) KOH, washed in PBS, blocked in 3% (w/v) bovine serum albumin (BSA) in 50 mM sodium phosphate−0.9% NaCl, pH 7.2, and then probed with antibodies using the method of Skerritt and Lew (1990). Antibody binding was detected with alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI). After probing with primary and alkaline phosphatase-conjugated secondary antibody, blots were incubated in 10 mL of chemiluminescent substrate [(3-(4-methoxyspiro (1,2-dioxetane-3,2″-(5″-chloro)tricyclo(3.3.1.1)decan-4-yl)]phenyl phosphate (CSPD, 0.045 mM in 0.1 M diethanolamine, 1 mM $MgCl_2$, pH 10) (BioRad) added to the membrane which was shaken for 5 min. Excess liquid was removed from the blot before sealing in a clear plastic bag, and the proteins were visualized using "Reflection" autoradiography film (DuPont, Melbourne, Australia). Using this method, membranes could be probed, stripped of antibodies, and reprobed. Blots were stripped by first washing in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl−0.05% (v/v) Tween 20 (TBST) followed by incubation in stripping solution (0.2 M glycine−HCl pH 2.2, 0.1% (w/v) SDS, and 1% (v/v) Tween 20) for 30 min. This wash was repeated two more times. The membrane was then washed twice for 5 min in TBST and reblocked with 3% BSA in PBS which prepared it for antibody probing. Individual rabbit polyclonal antisera (Sissons, M. J., Hill, A. S.; Skerritt, J. H. Unpublished) were used to probe the blots for detection of LMW-GS bearing N-terminal sequences of the LMW-m (METSRVPGL-), LMW-s (SHIPGLERPSGL-), alpha- and gamma-types.

Size Exclusion-HPLC. Size exclusion-HPLC analyses were performed using a Phenomenex Biosep SEP−SEC−S4000 column, 300 × 7.8 mm and a mobile phase of 50% acetonitrile−0.1% (v/v) trifluoroacetic acid (TFA). Experimental conditions were as follows—flow rate: 0.5 mL/min; wavelength: 214 nm; AUFS: 0.5; column temperature: 30 °C; and run time: 40 min. Chromatograms were captured using Beckman Gold Nouveau software, and the size distribution

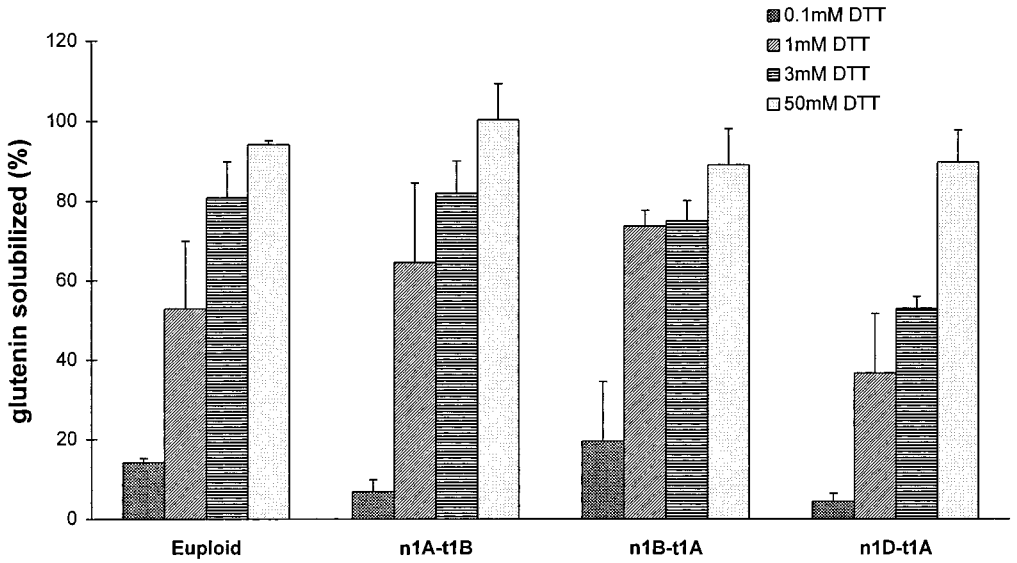

Figure 1. Solubilization of glutenin protein from Chinese Spring euploid flour and its nulli-tetra lines. Data were expressed relative to solubilization at high (100 mM) dithiothreitol. Data shown represents mean and standard deviation of two experiments conducted in triplicate.

was measured in two-min intervals using Design CAD 2D software, Version 7.0 (DesignCAD, Pryor, OK).

RESULTS AND DISCUSSION

Solubilization of the Glutenin Macropolymer. As the concentration of reducing agent increased, increasing amounts of protein was solubilized from the GMP of each flour. The protein concentration reached its maximum at the highest reductant concentration at which the GMP became fully reduced. In the Chinese Spring flours, most of the depolymerization of glutenin from the GMP occurred between 1 and 10 mM DTT, but differences in the subunit release characteristics were evident. Release of subunits from the nulli1B-tetra1A flour was greater than the other lines, particularly at low reductant concentrations. Each of the other lines had a more gradual pattern of protein solubilization (Figure 1).

Size Distribution of the Glutenin Macropolymer. Size-exclusion HPLC analyses of the protein extracted by stepwise reduction of glutenin macropolymer isolated from flours demonstrated that specific changes in the size distribution of the polymer occurred as reductant concentration increased (Figure 2A). As the reductant concentration increased, a greater total amount of protein was eluted (greater area under the profile), and there was a shift in the distribution of the profile toward later elution times, corresponding to lower molecular weight species. SDS−PAGE analyses, together with use of molecular weight markers (Larroque et al., 1996), indicated that the largest peak, centered at 23 min, corresponded to LMW-GS, while the smaller peak at 21 min corresponded to HMW-GS. Material eluting between 14 and 21 min thus corresponded to oligomers and polymeric glutenin. We were not able to calibrate the column at lower elution times due to the lack of availability of standards; however, the manufacturers claim that the exclusion limit is M_r 2 000 000.

At early stages of the reduction series, polypeptides corresponding in elution times to HMW- and LMW-GS were released from GMP, along with larger amounts of polymeric and oligomeric material (Figure 2A). As the reductant concentration increased there were fewer polymers but more oligomers and subunits, while at high reductant concentrations the oligomers broke down, resulting in the release of HMW and LMW subunits. The molecular weight distribution of the protein released by stepwise reduction, corresponding to larger polymers (retention time 14−16 min), smaller polymers (16−18 min), and oligomers (18−20 min), was analyzed by calculating the relative and absolute areas under the profile in 2-min increments. LMW-GS were released at lower reductant concentrations than HMW-GS and polymeric material (Figure 2A), but as the DTT concentration increased, HMW-GS were released, and depolymerization continued until the polymeric fraction broke down completely.

In euploid Chinese Spring flour, most of the depolymerization of the large polymers occurred at very low (<0.5 mM) DTT concentrations and most of the depolymerization of the smaller polymers occurred before 1 mM DTT. The majority of the depolymerization of the oligomers did not occur until 3−5 mM, with some oligomers remaining at 50 mM DTT. The depolymerization patterns in euploid and nulli1A-tetra1B Chinese Spring flour were similar, which was expected as they have similar HMW-GS composition. However, in both the nulli1B-tetra1A and nulli1D-tetra1A lines, which lack major HMW- (and LMW-) glutenin subunits, the polymeric fraction did not depolymerize until DTT concentrations were higher, thus depolymerization occurred over a narrower reductant concentration range (Figure 2B−C).

Depolymerization of Individual Glutenin Subunits. Although the increase in solubilized protein content as reductant concentration increased (measured by protein assay) was gradual and monotonic, SDS−PAGE analyses suggested that depolymerization of the GMP in flour occurred in three distinct stages (Figure 3). Banding patterns on stained gels clearly indicated that some subunits were released from GMP at low DTT concentrations in a fully reduced state and others in a partially reduced state (Figure 4A−B). Evidence for the presence of partially reduced subunits came from decreases in the mobilities of these subunits when the

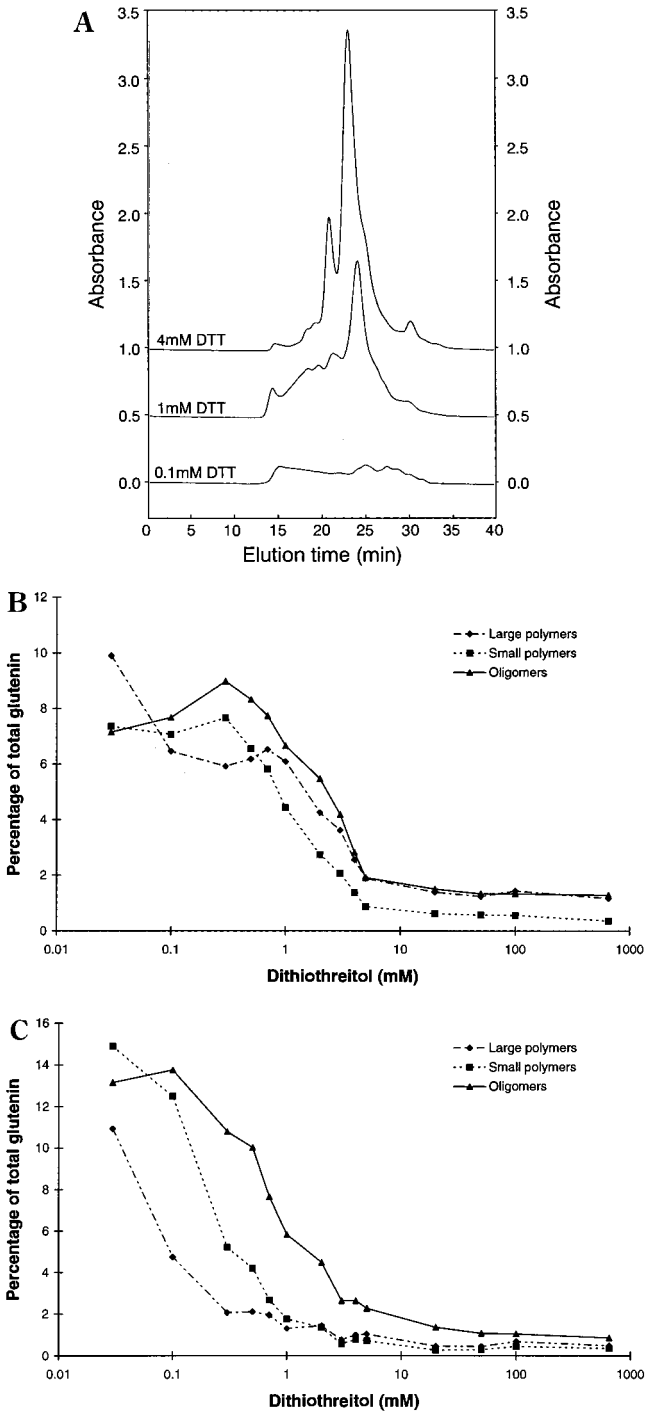

Figure 2. (A) Size-exclusion HPLC (SE-HPLC) profile of Chinese Spring euploid flour at different stages of reduction. Lower: 0.1 mM, center: 1 mM, upper: 4 mM DTT. Solubilization of oligomers and polymers from (B) Chinese Spring euploid flour and (C) n1B−t1A flour determined by SE-HPLC.

reductant concentration was increased, with the "tracking" of particular subunits in the complex mixture being facilitated by use of LMW-GS-specific antibodies and immunoblotting (Figure 4C−F). These subunits, which were primarily B-LMW-GS, were bound by intramolecular disulfide bonds, which were broken when the DTT concentration increased to 1−2 mM. The intramolecular bonding resulted in the subunits adopting a globular conformation, but upon complete reduction in the presence of SDS they would presumably adopt rodlike conformations, which would have lower and characteristic mobilities on SDS−PAGE. Evidence for this phenonemon also came from SE-HPLC (Figure 2A). The elution time of the largest peak, which corresponds to LMW-GS, is actually greater at intermediate reductant concentrations compared with fully reduced material. Densitometric analysis of SDS−PAGE gels has shown the polymeric fraction resisted breakdown to a greater extent in the euploid Chinese Spring flour than in the nulli1A-tetra1B, nulli1B-tetra1A, and nulli1D-tetra1A lines (Figure 3). An increase in the proportion of subunits was associated with polymer breakdown, and the rate at which subunits were released from the polymer was dependent on the resistance of the GMP to reductive dissociation. Generally, B-LMW subunits were released before either the HMW-GS or C-LMW-GS.

The general pattern of depolymerization indicated that HMW-GS had greater resistance to reductive dissociation from GMP than LMW-GS and C-LMW-GS more resistant than B-LMW-GS. This trend was observed on SDS−PAGE gel analyses of flours of euploid Chinese Spring and some of its nulli-tetra lines (Figure 4A−B). Some B-subunits were released from the polymer at DTT concentrations as low as 0.1 mM DTT. However, significant intramolecular bonding was retained, and these subunits did not become fully reduced until the DTT concentration approached 1−2 mM. When the subunits became fully reduced, their mobility on SDS−PAGE decreased as seen in Figure 4C−F. Release of C subunits did not occur as early as the B subunits, and a higher degree of intramolecular disulfide bonding existed within released B subunits as compared to C subunits. The B subunits were released earlier in the reduction series than other subunits in all Chinese Spring-derived lines. Furthermore, the change in the proportion of C subunit was not as significant as the B LMW subunits. C LMW-GS were released from the polymer as fully reduced subunits, or in a partially reduced state, becoming fully reduced when the DTT concentration approached 1−2 mM. It appears as though all LMW-GS that were released from the GMP at DTT concentrations less than 1−2 mM were associated with intramolecular disulfide bonding, and in all cases, the intramolecular disulfide bonds were broken once the DTT concentration exceeded this level.

The initial release of B-LMW-GS subunits occurred at 0.1−0.3 mM DTT. Although released from the GMP early, these subunits contained intramolecular disulfide bonds, which were slowly broken down over a wide DTT concentration range. However, complete reduction typically occurred before 20 mM DTT. The first high molecular weight dimers were not released from the GMP until 0.5−0.7 mM DTT, which was similar to the concentration at which the intramolecular bonds in the released B subunits had begun to break down. Release and subsequent reduction of the intramolecular disulfide bonds of C subunits took place over a wider DTT concentration range than B subunits. In addition to the release of subunits associated with intramolecular disulfide bonds, some C subunits appeared to be released in a fully reduced state. On SDS−PAGE gels there was not a shift in the apparent molecular weight of C-subunits which is indicative of conformational changes. Furthermore, C-subunits were not associated with the LMW-GS dimers. Release of these subunits occurred after 2 mM DTT was reached.

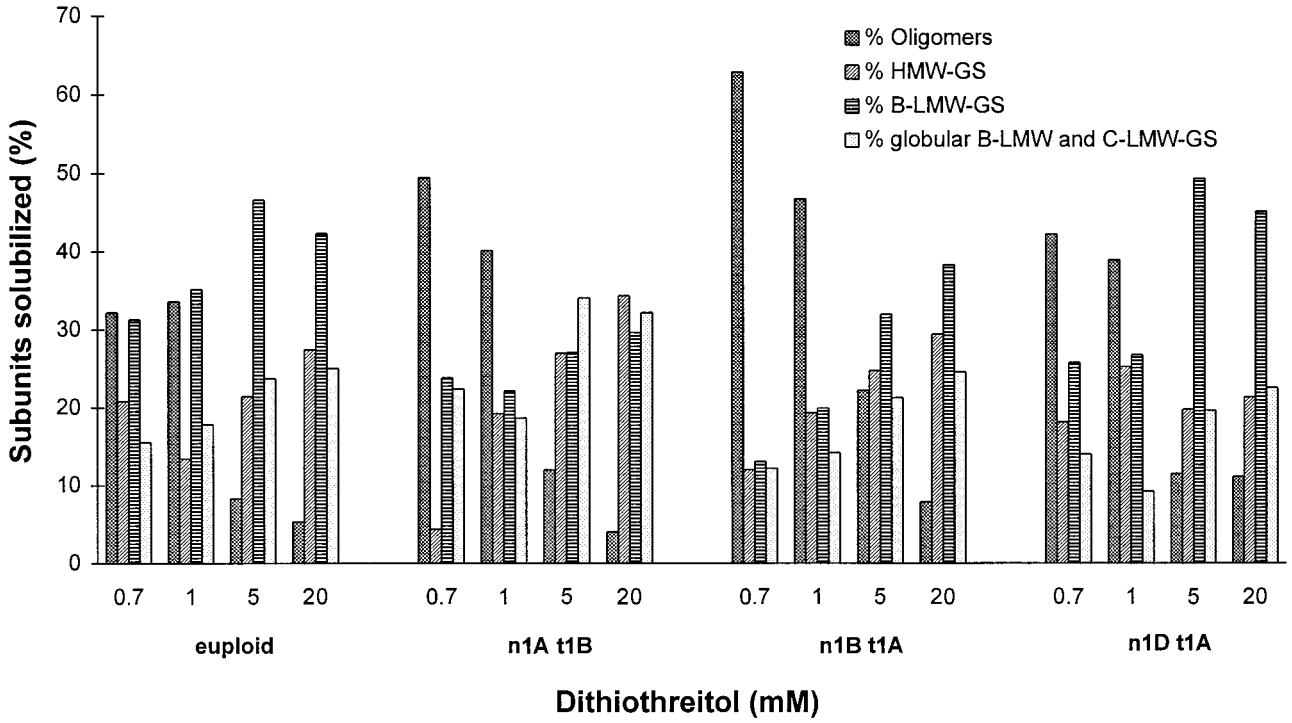

Figure 3. Release of oligomers and subunits of Chinese spring euploid wheat and its nulli-tetra lines determined by densitometric analysis of SDS−PAGE gels.

Release of Dimers from the Glutenin Macropolymer. As well as glutenin polymers, oligomers, and individual subunits, dimers consisting of either LMW-GS or HMW-GS were released during reduction of the glutenin macropolymer (Figure 3). The LMW dimers were comprised of B and C subunits, released between 0.1 and 0.3 mM DTT and with a molecular weight of approximately 90 000 (Figure 4C−F). Release of LMW dimers from the polymer occurred at a similar DTT concentration as that required to break intermolecular disulfide bonds of B subunits, and the dimers were reduced to monomers under the same reducing conditions (1−2 mM DTT) as required to break intramolecular disulfide bonds of the released subunits. In addition to the disulfide bonds associated with the B and C subunits and LMW dimers, dimers were released which were highly resistant to exposure to reducing agent (Figure 4A lane l, Figure 4B lane k). These dimers were composed of HMW subunits. Analysis of two-dimensional (partly reduced × fully reduced sample) SDS−PAGE gels showed that dimers of x−y and x−x type HMW-GS were present, but it does not appear LMW-GS were associated with these dimers (Figure 5). Subunits derived from the *Glu-D1* locus were associated with all dimers, while subunits derived from either the *Glu-A1* or *Glu-B1* loci were only associated with some of the dimers. Although some dimers did break down during reduction, they did not become fully reduced until the reducing agent concentration was high, and all other subunits (and dimers) had been released and fully reduced. The great resistance of these dimers indicates that HMW-GS may in fact form a "backbone" of the GMP.

Release of the first high molecular weight dimers from each of the Chinese Spring flours (euploid, n1A−t1B, n1B−t1A, n1D−t1A) occurred by 0.3 mM DTT; however, they were not fully reduced until the DTT concentration increased to 20 to over 100 mM. Dimers were reduced over a wide DTT range, but the concentration at which they were completely broken down into subunits was a function of their genetic composition and depended, to some extent, on the number and type of HMW-GS present. Dimers derived from the euploid Chinese Spring wheat resisted breakdown until the DTT concentration exceeded 100 mM; however, the dimers from nullisomic lines dissociated at lower reductant concentrations. In each flour one dimer of HMW-GS was observed that was highly resistant to breakdown (Figure 4A−B). Examination of the resistance of dimers from flours of different genetic lines has revealed that reduction of dimers occurs at different DTT concentrations. The resistant dimer from the euploid and n1D−t1A lines did not dissociate until the reductant concentration exceeded 100 mM. Dimers from n1A−t1B and n1B−t1A flours were less resistant to reduction, with the glutenin from the n1A−t1B flour becoming fully reduced by 50 mM DTT and that from the n1B−t1A flour becoming fully reduced by 20 mM DTT. The composition of the dimers released during reduction has shown that subunits derived from the *Glu-1D* locus are associated with a high proportion of the dimers. The dimers primarily consisted of *Glu 1Ax-Glu 1Dy, Glu 1Ax-Glu 1Ax, Glu 1Dx-Glu 1By*, and *Glu 1Dx-Glu 1Dy* derived subunits. The pattern of dimer dissociation indicates that dimers containing subunits derived from the *Glu-1D* locus are comparatively more resistant to reduction than dimers comprised of subunits which are derived from the *Glu-1A* or *Glu-1B* loci.

Role of Different Sequence Families. Release characteristics of subunits and dimers associated with the LMW-m (METSRVP), LMW-s (SHIPGLERPSGL), and alpha-type and gamma-type LMW-GS N-terminal amino acid sequences were examined in order to relate the release characteristics of these sequence types to the dissociation of the GMP during stepwise reduction (Figure 4C−F). In each Chinese Spring-derived flour, subunits containing the METSRVP N-terminal sequence were released by 0.1 mM DTT, but the subunits

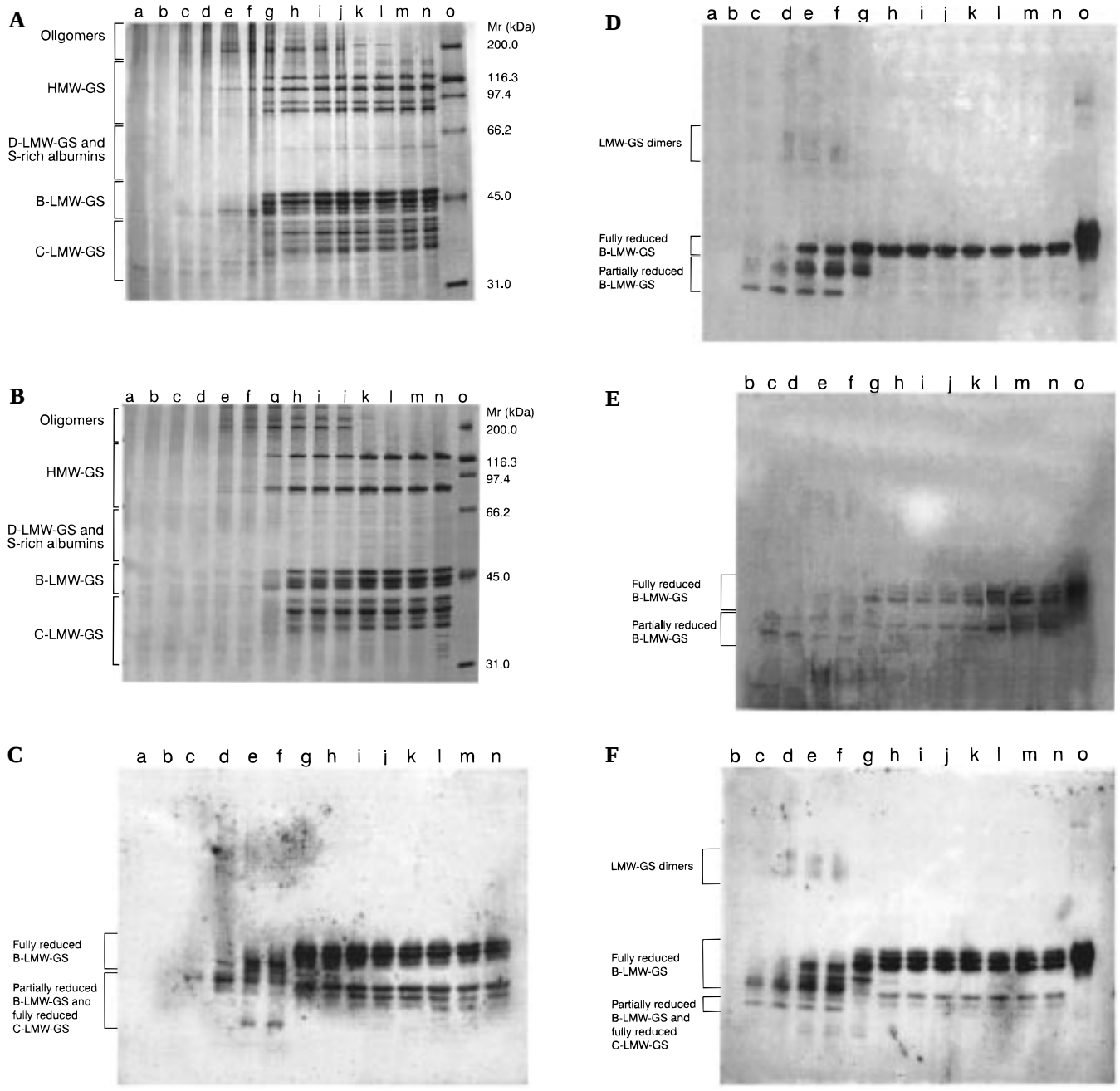

Figure 4. SDS−PAGE analysis of (A) Chinese Spring euploid flour, (B) Chinese Spring n1B−t1A flour, and immunoblots of Chinese Spring flour using antisera specific for different LMW-GS N-terminal sequence families, (C) SHIPGLERPSGL, (D) METSRVPGL, (E) alpha- and (F) gamma-LMW-GS. Lanes a−o: a, 0 mM DTT; b, 0.1 mM DTT; c, 0.3 mM DTT; d, 0.5 mM DTT; e, 0.7 mM DTT; f, 1 mM DTT; g, 2mM DTT; h, 3mM DTT; i, 4mM DTT; j, 5mM DTT; k, 20mM DTT; l, 50mM DTT; m, 100 mM DTT; n, fully reduced; o, molecular weight standards.

were intramolecularly bound, only becoming fully reduced when the reductant concentration reached 2 mM. However, the intramolecular disulfide bonds of these subunits were gradually broken between 0.3 and 2 mM. In addition to intramolecular bonds which result in the subunits having globular conformations (greater mobilities on SDS−PAGE gels dimers containing these subunits were released at 0.3 mM and broke down into fully reduced monomers at 2 mM). However, no LMW-GS dimers were detected in the n1D−t1A flour. Intramolecular bonds of the released subunits broke down by 2 mM DTT, which corresponded to the subunits becoming fully reduced. Dimers associated with the sequence type broke down at low reductant concentration, typically as low as 0.3 mM DTT. LMW-s subunits were released by 0.3 mM DTT and the intramolecular disulfide bonds fully reduced by 4 mM; dimers with this sequence were released and cleaved by 2 mM DTT. The subunits that were released were intramolecularly bound and became fully reduced by 5 mM DTT. Gamma-type subunits were released from the polymer between 0.1 and 0.3 mM DTT in each Chinese Spring line. In the euploid line, released subunits became fully reduced by 2 mM DTT, while subunits released from the nullisomic lines resisted breakdown until 4−5 mM DTT. In contrast however, the dimers from euploid and n1A−t1B flours were reduced to monomers by 0.7 mM, while the n1B−t1A and n1D−t1A lines did not become fully reduced until 2 mM DTT. Alpha-type subunits were released from the GMP over a wider reductant range than the

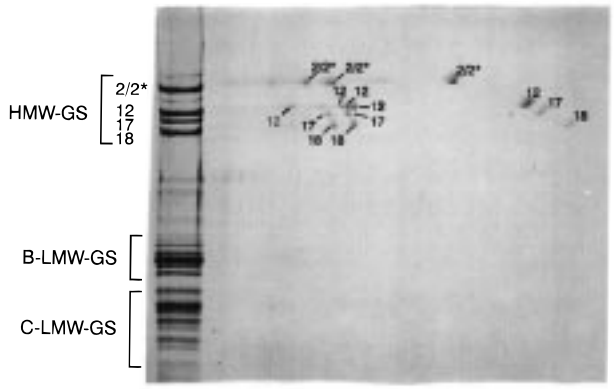

Figure 5. Two-dimensional SDS−PAGE of flour with glutenin subunit composition (b, i, a [2*, 17+18, 2+12]; b, b, b). The first dimension was reduced with 1 mM DTT and separated by 8% T SDS−PAGE, followed by complete reduction and separation by 12% T SDS−PAGE.

other sequence types studied. Subunits were initially released at 0.1 mM DTT, but the intramolecular bonds associated with these subunits were not fully reduced until 5−20 mM DTT.

Depolymerization of Glutenin Subunits in Doughs. The depolymerization behavior of glutenin subunits in doughs at different stages of mixing were examined and although the overall trends were similar to the Chinese Spring flour, differences in the depolymerization pattern were observed, particularly in overmixed doughs (Figure 6). It appears as though the mixing process results in changes in the susceptibility of different groups of subunits to reductive dissociation. During GMP breakdown, large polymers were released at lower reductant concentrations than small polymers, which in turn were released earlier in the reduction series than oligomers (Figure 7). Of particular interest were the differences in the rate of breakdown of the polymeric fraction of the GMP. Breakdown of the flour was more gradual than the doughs, with this fraction becoming completely reduced by 5 mM DTT, compared with 0.7−1 mM for each of the doughs. The oligomers and large polymers broke down over a wider DTT concentration range than the small polymers in each instance. Densitometric analysis of SDS−PAGE gels has demonstrated that the mixing process leads to changes in the GMP which results in differences in the sensitivity of the GMP to reduction (Figure 8). In overmixed doughs, the polymeric fraction makes up a larger proportion of the total glutenin fraction released at low reductant concentrations. Results indicates that changes occur during mixing that alter the structure of the GMP.

In general, HMW-GS had greater resistance to reductive dissociation from GMP than LMW-GS, and C-LMW-GS were more resistant than B-LMW-GS. Although this trend was consistent throughout the reduction series, variation did exist, particularly with doughs which had been overmixed, where a more random depolymerization pattern was observed (Figure 6A−B). The main differences in the depolymerization behavior of flour and dough was in the initial stages of reduction. Release of B-subunits containing intramolecular disulfide bonds, and their subsequent unfolding dominated the initial stages of depolymerization in flours. In overmixed doughs, depolymerization was less ordered, with HMW and LMW subunits being released at similar reducing agent concentrations. The breakdown of intramolecular bonds of B and C LMW-GS in doughs occurred at 0.7 mM DTT, whereas in flours these bonds resisted reduction until 1−2 mM DTT. In severely overmixed doughs, release of B and C subunits occurred simultaneously, and the degree of intramolecular bonding of the released subunits was minimal compared with flours. However, it should be noted that the greater solubilization of protein with SDS alone from overmixed dough (compared with flour) also leads to a higher reducing agent-to-protein ratio in the overmixed dough.

Differences in the depolymerization of subunits associated with the different N-terminal sequence types were also observed. Subunits containing the LMW-m (METSRVP) N-terminal sequence type were released during the SDS extraction step prior to exposure to reductant, and the release pattern and number of subunits released depended on mixing time (Figure 6C−D). Initial release of subunits containing the LMW-s (SHIPGLERPSGL) sequence type in doughs also occurred in the SDS extraction step. These subunits became fully reduced by 1−2 mM DTT, and dimers associated with these subunits became fully reduced by 0.3−0.5 mM DTT suggesting they have low resistance to reduction. Subunits of the gamma-type also did not resist reduction; the first subunits were released without exposure to DTT, and these became fully reduced by 0.5−0.7 mM DTT in each flour. Furthermore, dimers broke down by 0.1−0.3 mM DTT, demonstrating little resistance to reductive dissociation.

GENERAL DISCUSSION

Stepwise reduction of the GMP with dithiothreitol (DTT) resulted in the release of subunits, both in a fully reduced state and in a partially reduced state, dimers with an molecular weight of approximately 90 000, and higher molecular weight dimers with a molecular weight exceeding 200 000. Release of subunits and dimers was dependent on the reduction of intermolecular disulfide bonds, but as it became evident that the release of these proteins is not random, there must be a conformational arrangement which results in bonds between particular polypeptides being more susceptible to reduction than others. Identifying the order of depolymerization of subunits, dimers, and aggregates from the glutenin macropolymer assists in developing a better understanding of the protein structure. Changes in the size distribution, measured using SE-HPLC, were associated with release of aggregates, dimers, and monomers of the GMP during the reduction series. The reduction of the disulfide bonds, which are important to the structural integrity of the GMP, has been shown to have detrimental effects on dough mixing characteristics. These "rheologically effective" bonds are broken down at extremely low reductant concentration (Khan et al., 1994). Thus it is likely that at early stages of reduction the GMP breaks down into smaller sized aggregates which in turn become fully reduced with increased exposure to dithiothreitol. During the reduction series, polymeric fractions were released from the GMP, and with an increasing reductant concentration, the polymeric fraction became fully reduced. A consistent trend was observed during the reduction series; large polymers broke down more readily than small polymers, which in turn broke down more readily than oligomers. This shift in size distribution of the GMP resulted in the release of HMW- and LMW-GS. However, a shift

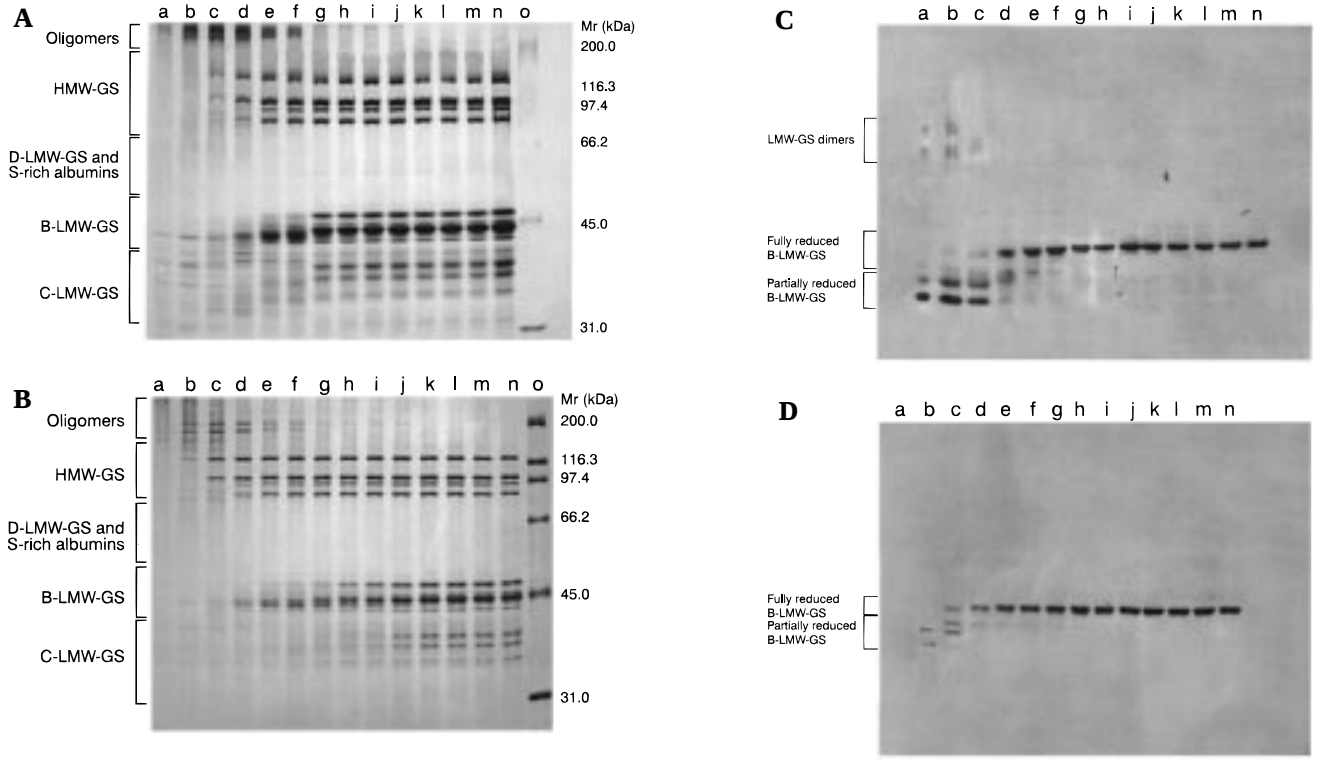

Figure 6. SDS−PAGE analysis of glutenin subunits released from (A) flour, (B) overmixed dough (2.25 × peak mix time), and immunoblots of glutenin subunits from (C) flour and (D) dough, probed using an antiserum specific for the LMW-m sequence family (METSRVP). Lanes a−o, dithiothreitol (DTT) concentrations as follows: a, 0 mM; b, 0.1 mM; c, 0.3 mM; d, 0.5 mM; e, 0.7 mM; f, 1 mM; g, 2 mM; h, 3 mM; i, 4 mM; j, 5 mM; k, 20 mM; l, 50 mM; m, 100 mM; n, fully reduced; o, molecular weight standards.

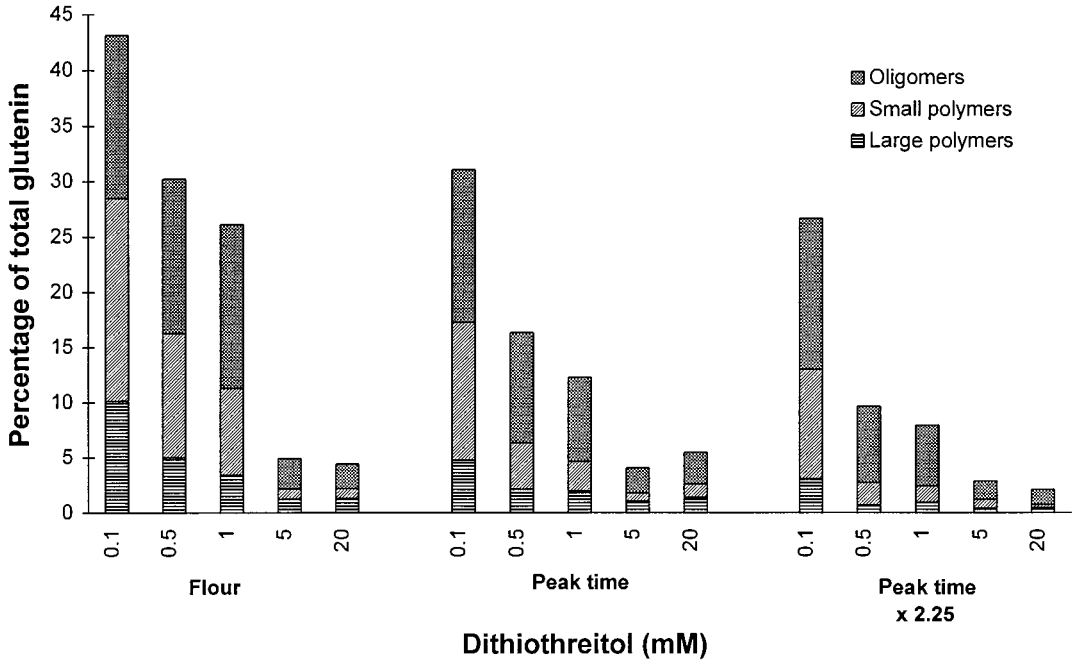

Figure 7. Solubilization of oligomers and polymers from flour and doughs (b, i, a [2*, 17+18, 2+12]; b, b, b) mixed to peak resistance and 10 min, determined by SE-HPLC. The proportion of the three polymeric groups was determined by calculating the area representing the polymeric fractions under the SE-HPLC profile in 2-min increments.

in retention times at the later stages of reduction indicates that intramolecular bonding is associated with the released subunits.

Release of subunits occurred in three distinct stages. First, weakly bound subunits and LMW-GS dimers were released, second, there was a change in conformation of subunits released at low DTT concentration and release of fully reduced subunits, and third, release of dimers highly resistant to reducing agent and subsequent breakdown of these dimers took place. These trends were consistent with all flours and doughs studied, and apart from overmixed doughs, the subunit release characteristics of the GMP was consistent with the polymer having a precise structural organization.

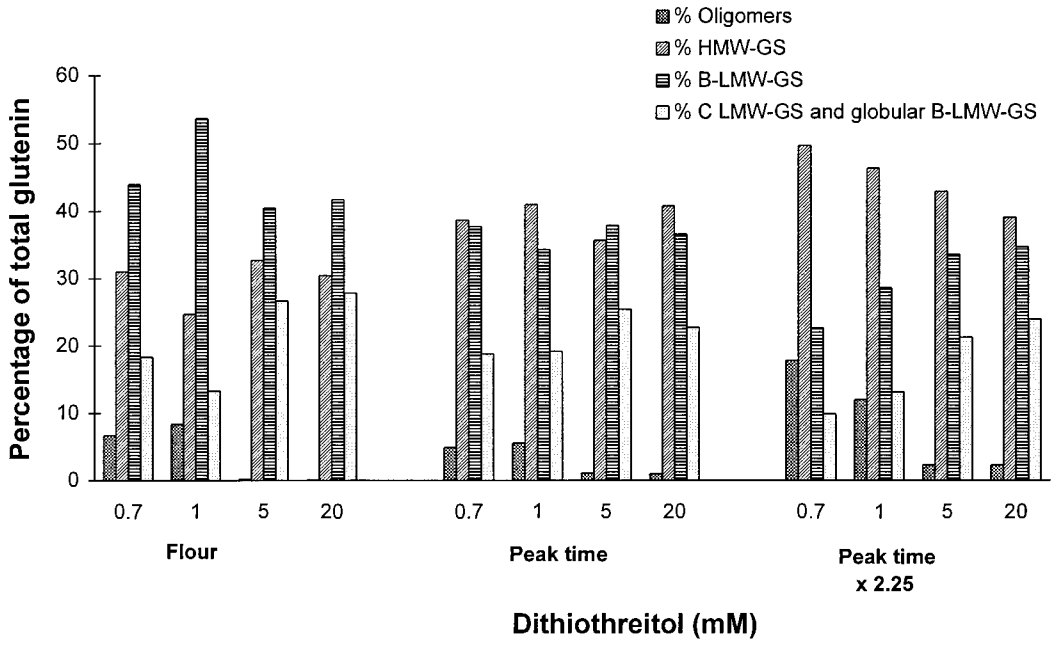

Figure 8. Release of oligomers and subunits of flour and dough (b, i, a [2*, 17+18, 2+12]; b, b, b) mixed to peak resistance and for 10 min determined by densitometric analysis of SDS−PAGE gels.

This study has focused on the role of disulfide bonding in terms of subunit release characteristics and possible influence of different alleles, N-terminal sequence types, and possible differences in structure between flours and doughs, particularly those which have been undermixed, overmixed, and mixed to peak development time.

The susceptibility of subunits to reduction with DTT varied considerably. According to Shewry and Tatham (1997), the rheological properties of a particular flour are influenced by the precise protein composition of the polymeric fraction. The composition of the polymeric fraction subsequently effects the molecular weight distribution of the polymer, density of covalent bonds, number of disulfide bonds between subunits, strength of bonds between subunits, and branching patterns of subunits. As a result, differences in the reduction of individual subunits from the polymer exist, particularly between flours with different HMW-GS composition. Previous studies using partial reduction techniques have shown that as the GMP breaks down subunits are released, which differ in susceptibility to reducing agent. During stepwise reduction, the proportion of LMW-GS released from the GMP increased as the polymeric fraction broke down (Werbeck and Belitz, 1988; Werbeck et al., 1989). These subunits, which are released at low reductant concentrations (primarily B-LMW-GS), often contain intramolecular bonds which become reduced as the reductant concentration increases (Matsumura et al., 1984). Kawamura et al. (1985) demonstrated that there is a selective cleavage of intermolecular bonds over intramolecular bonds at low reductant concentration which would result in subunits being released from the GMP before they are fully reduced. In the current study, B subunits were most sensitive to reductive dissociation and were released from the polymer at a low reducing agent concentration, and in some cases they were released as a result of the sample extraction process alone. When released at low reductant concentration however, these subunits were associated with a high degree of intramolecular bonding which only broke down when the reducing agent concentration increased. It is possible that these subunits are located toward the exterior of the polymer, and although they do contain many cysteine residues, many are involved with intramolecular disulfide bonds rather than intermolecular bonds. C-LMW-GS were released from the polymer over a wider reductant concentration range. The subunits released at higher DTT concentrations were fully reduced, while those released at low concentrations contained intramolecular disulfide bonds. As a result of the release patterns of these subunits, it is possible that they initially react with newly formed free sulfhydryl groups on the HMW-GS or B-LMW-GS and remain in the polymer at low reductant concentrations.

In addition to being released from the polymer as subunits, dimers of B-LMW subunits were also observed. These dimers had an approximate M_r of 90 000, and were associated with METSRVP, SHIPGLERPSGL, and gamma-type sequences. In addition, there has been some indication that B-LMW−C-LMW dimers also exist. These B subunits and dimers may provide support to the polymer, their breakdown resulting in decreased stability within the polymer leading to subsequent depolymerization and breakdown of the GMP. With respect to the depolymerization pattern as seen on SDS−PAGE gels, it appears as though release of B subunits is associated with polymer breakdown; once they have been released from the polymer, complete depolymerization follows. This not only may be due to the reductant concentration increasing to a critical level resulting in disulfide bonds associated with other subunits having greater exposure to reducing agent but may also indicate that the structural integrity of the polymer is influenced by these subunits. LMW subunits were released before the GMP broke down, and dimers or HMW subunits were released. LMW subunits were released at a slower rate than HMW subunits, but became fully released at a lower reductant concentration.

In addition to the release of LMW-GS dimers, HMW-GS dimers also exist, and it has been postulated that these dimers form a backbone to the polymer. If this is the case, it would be expected that the HMW-GS dimers resist breakdown to a greater extent than other subunits

(Werner et al., 1992; Kim and Bushuk, 1995). Earlier work has shown that the HMW dimers are composed of x−x and x−y type subunits (Lawrence and Payne; 1983, Kim and Bushuk, 1995), although Werner et al. (1992) were only able to identify homodimers of x-type HMW-GS. The HMW dimers may have a greater resistance to reduction as a result of a higher degree of disulfide bonding and may also be less available to reduction due to a higher degree of hydrophobicity (Kim and Bushuk, 1995), for example, x-type HMW-GS are more hydrophobic than y-type HMW-GS which may explain their greater resistance to reduction. Using two-dimensional electrophoresis, observations were made regarding the composition of the HMW dimers. Previously, Lawrence and Payne (1983) determined that the composition of oligomers is dependent on the HMW-GS composition of the flour. Subunits controlled by chromosome 1D were associated with a high proportion of the oligomers, while subunits controlled by chromosome 1B showed differences in their ability to associate to form dimers. A high proportion of subunits derived from chromosome 1D were also associated with dimers in this study. Both x−x and x−y type dimers were found to exist, but there was no evidence supporting the presence of y−y type dimers. The y−y dimers may exist, but as y subunits are thought to have greater water solubility characteristics, they may be more susceptible to reduction and are therefore released and fully reduced at an earlier stage of reduction (Kim and Bushuk, 1995). The HMW dimers were the last group of subunits to be fully reduced, and one dimer, in particular, resisted breakdown to a much greater extent than others. This dimer likely forms the central backbone of the polymer.

The chromosomal influence on the structural organization of the GMP was examined in an attempt to understand the possible role subunits derived from different loci have on the polymer structure. The main difference in the depolymerization of the GMP was that the resistant HMW dimer of the euploid wheat was better able to resist reduction than in the n1B−t1A wheat. The ability of the HMW dimer to resist reduction appears to be a function of the number and type of HMW subunits present. The n1B−t1A flour became fully reduced at 20 mM DTT, n1A−t1B became fully reduced by 100 mM, and n1D−t1A became fully reduced at a DTT concentration exceeding 100 mM. Likewise, the euploid wheat did not become fully reduced until the DTT concentration exceeded 100 mM. Subunits 7 and 8 (encoded on chromosome 1B) are relatively more hydrophilic (Kawka et al., 1992; Magnus and Khan, 1992), and it would therefore be expected that they would be more available to reduction in a hydrophilic environment such as the conditions of the experiment. Since subunits controlled by chromosome 1B have demonstrated differences in their ability to associate with subunits controlled by other chromosomes (Lawrence and Payne, 1983), it may be possible that the interaction of these subunits with more hydrophobic subunits results in a structural arrangement that prevents rapid reduction of these oligomers.

The dough mixing process may lead to changes in the solubility of the glutenin proteins. As a result, more glutenin proteins are washed out of the dough as compared to flour during the extraction procedure. However, there were not significant changes in the depolymerization pattern, except in severely overmixed doughs. The primary difference was in the rate of subunit release. The GMP in doughs began to depolymerize at lower reductant concentration, but the concentration at which the HMW dimers completely broke down did not differ. These results suggest that although the structure of the GMP may change due to changes in solubility, the disulfide bonded status is not affected to the same extent, except in severely overmixed doughs. Despite the complexity of the GMP, consistent trends during stepwise reduction provide further evidence that a specific structural organization exists within the glutenin macropolymer of wheat flour. The depolymerization of glutenin during dough over-mixing and by stepwise chemical reduction may have similar effects. During dough mixing and breakdown, the amount and average molecular weight of the glutenin macropolymer also decreased (Skerritt et al., 1994, 1996). Depolymerization of LMW-GS commenced earlier than HMW-GS, while most B-LMW-GS depolymerized earlier than C-LMW-GS, which enrich in the "debranched" glutenin polymer.

ABBREVIATIONS USED

CSPD, 3-(4-methoxyspiro(1,2-dioxetane-3,2″-(5″-chloro)-tricyclo(3.3.1.1)decan-4-yl)phenyl phosphate; DTT, dithiothreitol; GMP, glutenin macropolymer; HMW-GS, high molecular weight glutenin subunit; HPLC, high performance liquid chromatography; LMW-GS, low molecular weight glutenin subunit; PVDF, polyvinylidine difluoride; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST, tris buffered saline Tween; 4-VP, 4-vinyl pyridine.

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