Composition of High-Molecular-Weight Glutenin Subunit Dimers Formed by Partial Reduction of Residue Glutenin[1]

WILLIAM E. WERNER,[2,3] A. ELVA ADALSTEINS,[2] and DONALD D. KASARDA[2,4]

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ABSTRACT

Partial reduction of residue (glutenin) proteins remaining after extraction of flour with sodium dodecyl sulfate (SDS) solution produced soluble monomers, dimers, and possibly trimers of high-molecular-weight glutenin subunits (HMW-GS) and monomers of low-molecular-weight glutenin subunits as indicated by SDS-polyacrylamide gel electrophoresis (PAGE). The partially reduced proteins were soluble in the dithiothreitol-Tris buffer (pH 8.5), lacking SDS, that was used to reduce the residue proteins. The disulfide-linked subunits in the dimers were analyzed by two-dimensional SDS-PAGE with reducing agent present only in the second dimension. The two-dimensional analyses indicated that pairs consisting of an x-type and a y-type HMW-GS predominated in the dimers produced at low levels of reducing agent (for example, 0.5 m*M* dithiothreitol). Dimers composed only of x-types were sometimes observed, particularly at higher levels of reducing agent. No dimers combining only y-type HMW-GS were noted. The faster SDS-PAGE mobilities of y-type monomers relative to the mobilities of the equivalent, completely reduced forms indicated a more compact structure for the partially reduced forms, presumably due to the presence of at least one intramolecular disulfide bond. The intramolecular disulfide bonds of the y-type HMW-GS monomers and the combinations of HMW-GS in the dimers of HMW-GS are likely to reflect arrangements found in native glutenin.

The glutenin fraction of wheat (*Triticum aestivum*) storage proteins is an essential contributor to the viscoelastic properties of wheat flour doughs (Wall 1979). Some years ago it was recognized that the addition of reducing or sulfhydryl reagents caused the breakdown of dough structure through loss of dough elasticity (Pence and Olcott 1952, Mecham et al 1963). These observations led to the hypothesis that intermolecular disulfide bonds are essential to the viscoelastic properties of doughs, which became the foundation for models developed to account for the viscoelastic and gas-holding capabilities of doughs (Ewart 1968, 1990; Khan and Bushuk 1979; Graveland et al 1985). It is now generally accepted that glutenin protein subunits are joined by intermolecular disulfide bonds to form high-molecular-weight complex molecules, which we shall also refer to as glutenin polymers. These polymers are of various sizes, depending on the number of subunits incorporated into a given molecule, which may range from just two to many subunits (Kasarda 1989). The molecular masses of glutenin polymers range from about 80,000 Da (80 kDa) up to several million daltons (although the upper range is not well defined).

Glutenin polymers are constructed of two main types of subunits, which were originally classified according to their electrophoretic mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under fully reduced conditions (Payne and Corfield 1979). The subunits with the slowest mobilities are often referred to as the high-molecular-weight glutenin subunits (HWM-GS) and the group with faster mobilities as the low-molecular-weight glutenin subunits (LMW-GS). Extensive genetic and biochemical analysis of wheat varieties has revealed the presence of approximately 20 distinct proteins that migrate in the HMW-GS region (Lawrence and Shepherd 1980, Payne et al 1981a, Shewry et al 1989). Many of these proteins are products of allelic genes, and normally not more than five HMW-GS are expressed in a given cultivar.

The genes coding for HMW-GS have been mapped (Bietz et al 1975) to the long arms of the homoeologous group 1 chromosomes (1A, 1B, and 1D), where pairs of linked genes on each chromosome potentially code for two distinct proteins. These two different proteins are designated x-type and y-type on the basis of compositional and structural differences (Payne et al 1981b, Shewry et al 1989). In any given wheat variety, the 1A chromosome expresses either one (x-type) or no HMW subunit, the 1B chrom-

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[2]U.S. Department of Agriculture, Agricultural Research Service, Western Regional Research Center, Albany, CA 94710.
[3]Present address: Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404.
[4]Author to whom correspondence should be addressed.

osome expresses either one (x-type) or two (one x- and one y-type) HMW subunits, and the 1D chromosome almost always expresses two HMW subunits (one x-type and one y-type). The allelic variation within the HMW-GS has been found to correlate significantly with differences in bread-making quality of wheat varieties (Payne et al 1981a, Payne 1987); for example, the pair of HMW glutenin subunits 1Dx5 and 1Dy10 correlates with good quality, and the allelic equivalent pair 1Dx2 and 1Dy12 correlates with poor quality.

The very large sizes of most glutenin polymers make the complete solubilization of native glutenin extremely difficult. Nevertheless, solvents containing SDS or acetic acid have been shown to extract much of the native (unreduced) glutenin (Bietz and Wall 1975, Field et al 1983, He and Hoseney 1991). Gel permeation chromatography has been used extensively to fractionate soluble glutenin polymers (Payne and Corfield 1979; Graveland et al 1982, 1985; Field et al 1983). When the various fractions were sampled and analyzed by electrophoresis under reducing conditions, most contained both HMW- and LMW-GS, but the lowest molecular-weight range or fractions soluble in 70% ethanol contained only LMW-GS. Earlier studies involving electrophoretic analysis of partially reduced glutenin demonstrated the existence of dimers (Lawrence and Payne 1983, Graveland et al 1985), and perhaps trimers (Graveland et al 1985), consisting solely of HMW-GS. Additionally, a HMW-GS (x-type), derived from a cloned gene expressed in bacteria, polymerized into disulfide-linked oligomers as indicated by SDS-PAGE patterns (Galili 1989).

In this study, SDS-PAGE was employed to characterize partially reduced glutenin to obtain additional information about the subunit and disulfide arrangements of native glutenin. Both one- and two-dimensional (unreduced versus reduced) SDS-PAGE were used to show the presence of HMW-GS dimers and possibly trimers in partially reduced glutenin and to show the subunit composition of the dimers. Furthermore, information about the presence of intramolecular disulfide bonds in HMW-GS monomers was inferred from the differences in mobility between partially and fully reduced monomers of HMW-GS.

MATERIALS AND METHODS

Wheat Cultivars and Lines

Grain of the wheat cultivars Yecora Rojo (good baking quality), lot CWC-141, and Anza (poor baking quality), lot CWC-150, was obtained from the California Wheat Commission. Chinese Spring was supplied by J. G. Waines, University of California, Riverside, and increased by C. O. Qualset and H. Vogt, University of California, Davis. These varieties possess different combinations of HMW subunits: 1, 5, 17, 18, and 10 for Yecora Rojo and 2, 7, 8, and 12 for Anza and Chinese Spring (using the numbering system of Payne et al [1984]). Subunit 1 is coded by a gene on chromosome 1A (1Ax1); subunits 5+10 are allelic with subunits 2+12 and are coded on chromosome 1D; and subunits 17+18 are allelic with subunits 7+8 and are coded on chromosome 1B.

The grain was milled into flour with a Brabender Quadrumat Senior mill and the endosperm fraction (white flour) was used for electrophoretic analysis. The Yecora Rojo flour had 11.0% protein; the Anza flour had 10.0% protein; and the Chinese Spring flour had 11.1%, on an "as is" basis by Kjeldahl nitrogen analysis ($N \times 5.7$).

The aneuploid lines of Chinese Spring used for analysis of potential disulfide bond recombinations were nullisomic-tetrasomics (n-t) in which a particular chromosome was missing and was compensated for by two extra doses of a homoeologous chromosome (Sears 1954). In this study, lines n1B-t1A and n1D-t1B were used. For protein extractions of the n-t materials, whole grain was folded in glassine paper, crushed with a hammer, placed in a microfuge tube, and extracted with added solvent.

Partial Reduction

In a typical experiment, 1 g of flour was gently stirred for 45 min with 40 ml of Buffer A (50 m*M* Tris-HCl, pH 8.5, 2% SDS). The residue was collected by centrifugation (14,000 × *g*), washed once with a 20-ml portion of water and twice with 20-ml portions of Buffer B (50 m*M* Tris-HCl, pH 8.5, no SDS). The residue proteins from an SDS extraction consist almost entirely of glutenin subunits (Graveland et al 1982). For partial reductions, the washed residue (usually from 1 g of flour) was resuspended in 5–10 ml of Buffer B containing various concentrations of dithiothreitol (DTT) and agitated gently overnight on a rotating platform. In a few experiments, more vigorous agitation or magnetic stirring of the residue was performed. A time course for partial reduction was also studied. After partial reduction, the supernatant was clarified by centrifugation at about 14,000 × *g* and used as is for electrophoretic analysis. Samples were sometimes mixed with 2% SDS before electrophoretic analysis; this did not appear to affect the electrophoretic patterns.

Partial reduction of protein from nullisomic-tetrasomic lines. For the experiment with the n-t lines, the procedure was similar to that described above except that whole grain was used rather than flour; 400 mg of n1B-t1A was ground together with a 200-mg portion of n1D-t1B grain, extracted with 10 ml of Buffer A, and washed with 10-ml portions of water and Buffer B. The residue was then partially reduced with 2 ml of 0.6 m*M* DTT for electrophoresis as described above.

Effect of extractant volume on dimer concentration. To study the effect of protein concentration in the supernatant solution on the amount of dimers produced by partial reduction of residue, equal portions of washed residue were treated with different volumes of buffer over a 15-fold range, all 0.6 m*M* in DTT. The relative amounts of protein present in the supernatant solutions (which increased as volume of reductant solution decreased) were estimated by measuring the absorbance at 276 nm. No correction was made for the absorbance of oxidized DTT, which should be constant.

Electrophoresis

Electrophoresis was performed as a modification of the method of Laemmli (1970). The Protean I apparatus (Bio-Rad, Richmond, CA) operated at constant current (25 mA per 0.75 mm of gel) was employed in this study. The stacking gel was 3.8% acrylamide monomer that included 1.35% bis-acrylamide cross-linker, and the separating gel was 12% acrylamide monomer that included 1.35% bis-acrylamide cross-linker and 12% glycerol. The glycerol increased the density of the solution, thereby allowing the stacking gel to be poured on top of the running gel before polymerization of the acrylamide. Sample solutions that did not contain SDS were mixed with about a one-third volume of 2% SDS for loading on gels. After electrophoresis, the gels were stained overnight with 0.05% Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO) in 5% ethanol and 6% trichloroacetic acid or by the method of Neuhoff et al (1988), in which colloidal Coomassie Brilliant Blue G-250 (Sigma) was used (without the fixation step). To obtain a larger pore size that might resolve oligomers greater than trimers, SDS-PAGE was also performed with 9 and 10% acrylamide gels and proportionately less bis-acrylamide. SDS-PAGE gel calibration for determination of molecular weights was done with the following standards: myosin (mol wt 205,000), β-galactosidase (mol wt 116,000), bovine serum albumin (mol wt 66,000), ovalbumin (mol wt 45,000), carbonic anhydrase (mol wt 29,000), and cytochrome C (mol wt 13,000).

A video densitometer (Bio-Rad) was used in the transmittance mode to quantify the relative amounts of HMW dimers, HMW monomers, and LMW monomers in each of the stained gels relative to one another. In addition, the absolute areas under the complete set of dimer peaks and the complete set of monomer peaks were determined.

Two-dimensional gel electrophoresis. For two-dimensional gels, the partially reduced glutenin was loaded onto a 12% gel in the absence of 2-mercaptoethanol and subjected to electrophoresis (first dimension). Next, a lane was cut from the gel and equilibrated with a buffer containing 5% 2-mercaptoethanol for 15 min. The gel slice was then laid on top of another 12% gel, and the second dimension was subjected to electrophoresis. In some experiments,

the gel was stained according to the procedure of Neuhoff et al (1988). Then the desired part of a pattern was cut out after staining, equilibrated as above, placed on the second dimension, and subjected to electrophoresis. The staining did not interfere with the second-dimension analysis. Reference strips from the first dimension were saved to be included in the photographs of the second-dimension gels. These reference patterns do not coincide exactly in mobilities with the protein spots of the second dimension because of swelling of the second-dimension gel.

RESULTS

Solubility of Glutenin Subunits After Exposure to SDS

Normally, glutenin remaining after extraction of flour with SDS solution would be expected to have minimal solubility in Tris buffer at pH 8.5, but in this study the supernatant solutions from the partial reductions of residue had moderately large protein concentrations of about 2 mg/ml. After extraction of the residue remaining from the partial reduction with normal reducing buffer, a relatively faint pattern upon SDS-PAGE analysis of the extract indicated that much of the glutenin had been extracted. The increased protein solubility in our experiments was apparently due to the prior complexation of the glutenin subunits with SDS. Some preliminary experiments had indicated less protein solubility for an equivalent amount of reducing agent when the residue was prepared by extraction with other prolamin solvents.

Partial Reduction of Residue Proteins—One-Dimensional SDS-PAGE Analysis

Figure 1 shows one-dimensional SDS-PAGE patterns of partially reduced residue proteins from Yecora Rojo (lanes a–d) and Chinese Spring (lanes g–i), analyzed without reducing agent in the SDS sample and running buffers, compared with the patterns of fully reduced flour proteins from the two varieties (lanes e and f). When reduction was done with 0.05 m*M* DTT (Fig. 1, lanes a and j), most of the protein solubilized was too large to enter the gel, as indicated by heavy slot staining, which is in accord with the results of Ng et al (1991) for low levels of reduction. Some bands in the patterns corresponded in mobilities to dimers of HMW-GS as described by Lawrence and Payne (1983), an assignment we confirmed by two-dimensional analysis (see later figures and discussion below). Also present were bands that seemed likely to correspond to trimers of HMW-GS, although this assignment is somewhat arbitrary. In each of our experiments, including some with low levels of reducing agent and some analyses in 9% gels (results not shown), the apparent trimers were only faintly stained in the gels, whereas the amounts of dimers were often substantial, as seen from their staining intensities (Fig. 1, lanes c and h). Only the faintest traces of bands corresponding in mobilities to monomers of HMW-GS were present at 0.05 m*M* DTT in the patterns of Fig. 1, but LMW-GS monomers were evident. Thus, HMW-GS dimers and LMW-GS monomers were formed by partial reduction before any HMW-GS monomers.

Increasing the DTT concentration to 0.5 m*M* led to a decrease in slot material and an increase in the intensities of bands corresponding to dimers and monomers of HMW-GS and monomers of LMW-GS (Fig. 1, lanes b and i). This trend continued with increase in the DTT concentration to 1.0 m*M* (Fig. 1, lanes c and h). At a DTT concentration of 5 m*M*, the dimers had become faint relative to monomers and there was little evidence of slot material (Fig. 1, lanes d and g), which indicated that all glutenin polymers beyond the dimer level had been reduced. In other experiments (results not shown), however, we observed a qualitative change in the dimer pattern at DTT concentrations higher than those used in this study, along with a decrease in the intensity of the dimer pattern. Although partial reduction was usually done for about 12 hr, a time series with 0.6 m*M* DTT indicated that even 15 min of partial reduction gave a pattern qualitatively similar to that of Fig. 1, lane b, but of reduced intensity.

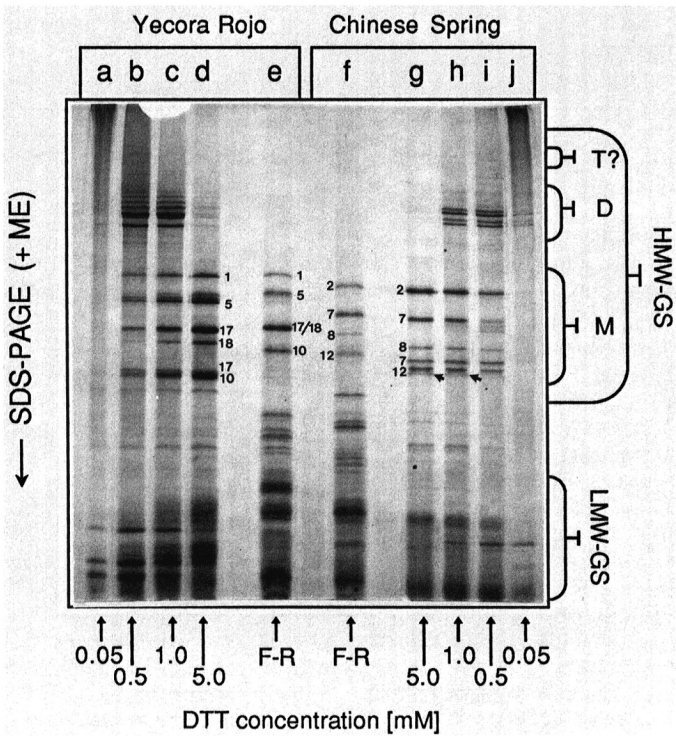

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of partially reduced SDS residues of Yecora Rojo (lanes a–d) and Chinese Spring (lanes g–i) flours. Dithiothreitol (DTT) concentrations used for each sample are indicated at bottom; F-R indicates fully reduced seed extracts (lanes e and f). Regions of mobility corresponding to low-molecular-weight glutenin subunits (LMW-GS) and to monomers (M), dimers (D), and putative trimers (T?) of high-molecular-weight glutenin subunits (HMW-GS) are bracketed. Payne numbers for various HMW-GS are indicated on the pattern; subunits 17 and 18 failed to resolve on this particular gel. An artifact that occurred only on this gel near CS subunit 12 is indicated by arrowheads in lanes g and h. ME = mercaptoethanol.

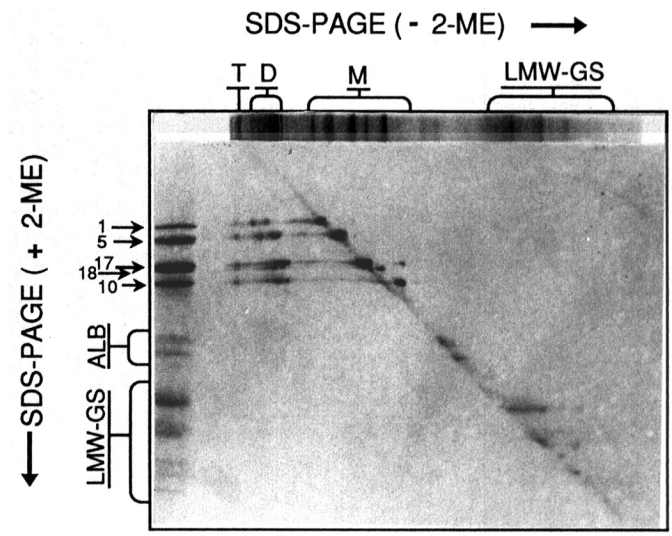

Fig. 2. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of partially reduced residue glutenin from Yecora Rojo. First dimension without added mercaptoethanol (− ME); second dimension with mercaptoethanol (+ ME). Regions of mobility corresponding to low-molecular-weight glutenin subunits (LMW-GS) and to monomers (M), dimers (D), and trimers (T?) of high-molecular-weight glutenin subunits (HMW-GS) are bracketed. Also shown are monomers of aggregated β-amylases (ALB) (Gupta et al 1991). Payne numbers for the fully reduced HMW-GS are given with arrows pointing to appropriate bands in the reference (fully reduced residue) pattern in the second dimension.

Interpretation of Two-Dimensional SDS-PAGE Patterns

Because reference must be made to both one- and two-dimensional gel patterns in describing results, we mention briefly here some aspects of these patterns. Proteins having the same mobilities whether reduced or unreduced (or, in this case, partially reduced) formed a diagonal line in the patterns. Proteins with greater mobilities in the first dimension (no added reducing agent) than in the second dimension (proteins fully reduced) had a more compact conformational structure in their partially reduced forms as a consequence of having intact intermolecular disulfide bonds (Graveland et al 1985, Kawamura et al 1985, Bulleid and Freedman 1988); these proteins appeared above (to the right of) the diagonal in the patterns. Polymeric or oligomeric proteins with subunits linked by intermolecular disulfide bonds, when reduced for the second dimension, produced the constituent subunits; these subunits moved more rapidly than the parent oligomers (usually dimers here) and so appeared below (to the left of) the diagonal in the two-dimensional patterns.

Partial Reduction of Residue Proteins—Two-Dimensional SDS-PAGE Analysis

Even at higher DTT concentrations, the patterns of the apparent monomers of HMW-GS (Fig. 1) differed from the patterns of fully reduced HMW-GS in one-dimensional gels. In the two-dimensional patterns (Figs. 2–4), the partially reduced forms of y-type HMW-GS subunits exhibited significantly greater mobilities than their fully reduced forms in SDS-PAGE analysis. Furthermore, usually no (or only small amounts of) fully reduced y-type subunits were evident.

Fully reduced subunit 1Dy12 of Chinese Spring migrates slightly faster than fully reduced subunit 1Dy10 of Yecora Rojo (see Fig. 1, lanes e and f) even though subunit 1Dy12 has a slightly larger molecular weight from sequence analysis (Greene et al 1988). In the partially reduced form, however, subunit 1Dy10 migrates substantially faster than partially reduced subunit 1Dy12 (Fig. 1, lanes d and g). Similarly, subunits 1By8 and 1By18 existed almost solely in high-mobility forms when partially reduced, but the mobility changes produced upon complete reduction were about the same for these two subunits (Figs. 1–3) and smaller than the change for 1Dy10. These 1B-coded subunits had mobility changes about the same as that for subunit 1Dy12.

Subunits 1Ax1 and 1Dx5 (x-types) of Yecora Rojo usually exhibited essentially the same mobilities, whether partially reduced or fully reduced (Fig. 2), although partially reduced subunit 1Dx5 gave three closely spaced bands (Fig. 1) with the fastest band being predominant at low levels of reductant and the slowest being predominant at higher levels of reductant. Subunit 1Bx7 apparently gave three closely spaced bands at 0.5 m*M* DTT, but in this case the faint bands leading that of 1Bx7 may represent faster-moving forms of subunit 2 (see discussion following and Fig. 3).

Much faster-moving x-type subunit monomers (partially reduced) were occasionally seen simultaneously with monomers of normal mobilities (presumably fully reduced) for all x-type subunits (note subunits 1Bx17 [Fig. 2], 1Dx2 [Fig. 3], and 1Bx7 [Fig. 3], which had components both on and above the diagonal). These more-compact forms of all x-type subunits were considerably faster than the faster-moving forms of the partially reduced y-type subunits so that the differences in mobility between the partially reduced form and the fully reduced form were in the ratio of approximately 3:2:1 for all x-types, 1Dy10, and all By-types plus 1Dy12, respectively. The decreases in apparent molecular weights for the fast-moving partially reduced forms of selected Yecora Rojo subunits relative to those of fully reduced forms (determined from an SDS-PAGE gel that included standards) were: 1Dx17, from 102,000 to 88,000 (14%); 1Dy18, from 98,000 to 93,000 (5%); and 1Dy10, from 91,000 to 82,000 (10%).

Effects of Agitation

A few experiments were made with more vigorous agitation or with magnetic stirring of the residue during partial reduction. This appeared to enhance the formation of the faster-moving x-types, as can be seen in Fig. 4. Vigorous magnetic stirring resulted in the x-type monomers of Yecora Rojo appearing only in their fast-moving forms (Fig. 4A), whereas vigorous agitation without magnetic stirring resulted in the presence of both normal (diagonal) and fast-moving forms for the x-type components (Fig. 4B).

Compositions of HMW-GS Dimers

The compositions of the HMW-GS dimers of the three varieties studied were analyzed by two-dimensional SDS-PAGE as typified

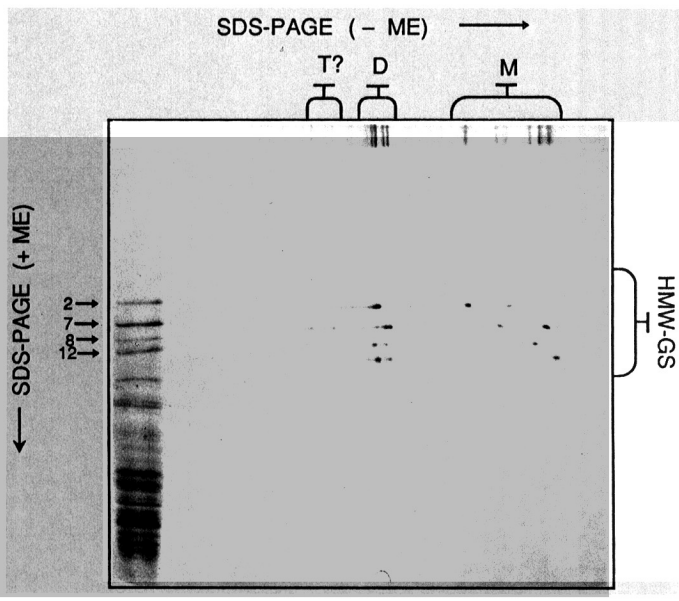

Fig. 3. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of partially reduced residue glutenin from Chinese Spring. First dimension without added mercaptoethanol (– ME); second dimension with mercaptoethanol (+ ME). Regions of mobility for the first dimension corresponding to monomers (M), dimers (D), and putative trimers (T?) of high-molecular-weight glutenin subunits (HMW-GS) are bracketed. Payne numbers are indicated for the fully reduced HMW-GS in the second dimension. Only monomers and oligomers of HMW-GS are included in the two-dimensional analysis because the first dimension analysis was sufficiently long that other proteins migrated off the gel.

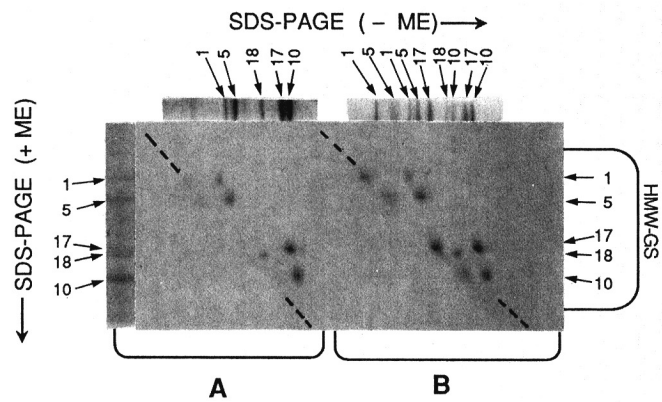

Fig. 4. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of partially reduced residue proteins (0.5 m*M* dithiothreitol) from experiments in which residue from Yecora Rojo flour was stirred vigorously by a magnetic stirring bar during reduction (A) or agitated vigorously on a rotating platform during reduction (B). Strips including only the high-molecular-weight glutenin subunits (HMW-GS) monomers were cut out from the first-dimension gel and analyzed simultaneously on a single second-dimension gel. The Payne numbers of the partially reduced and fully reduced HMW-GS are indicated. The positions of the diagonals (corresponding to proteins with no difference in mobility in first and second dimension—not obvious in the A pattern) are approximated by dashed lines.

in the patterns of Figs. 2 and 3. On top of each gel is a lane from a first-dimension gel showing the pattern of the partially reduced residue proteins, while on the left is the original sample after complete reduction (Fig. 2) or a fully reduced flour extract (Fig. 3). For the material entering the gel, only HMW-GS were found below (to the left of) the diagonal. This suggests that the reducing conditions employed cleaved all the intermolecular disulfide bonds between LMW-GS and HMW-GS and between the LMW-GS themselves, leaving dimers of HMW-GS as the main disulfide-linked forms.

There were two main clusters of HMW-GS below the diagonal in the patterns of Figures 2 and 3, corresponding to dimers and (probably) trimers. We were not able to analyze the slower moving cluster, but the dimers were resolved well enough so that compositions of the major bands could be determined from the two-dimensional patterns. Proceeding from right to left in Figure 2 (and in other patterns not shown), the subunit pairs that could be discerned in the Yecora Rojo pattern were as follows: 17-10, 17-18, 5-10, 5-18, 1-10, 1-18, 5-5, and 1-1. There was no evidence of y-y dimers, which we would expect to run faster than (appear to the right of) the 17-10 dimer in the first dimension in Fig. 2 because they should have lesser molecular weights. We were not able to recognize the combination 17-17, although both 1-1 and 5-5 (apparent) dimers were present, possibly because of lack of adequate resolution and the general background streaking that was always present. Only homodimers of x-types were observed with no mixed x dimers apparent (1-5, 1-17, or 5-17).

In Figure 3, the compositions of the strongest dimers from Chinese Spring were as follows (again from right to left): 7-12, 7-8, 2-10, and 2-8, that is, all combinations of x-type and y-type subunits. Anza was not analyzed by the two-dimensional approach; its one-dimensional pattern appeared identical to that of Chinese Spring.

We did not observe x-x dimers in every experiment (Fig. 3). Analysis of dimers from an experiment with Yecora Rojo in which the DTT concentration was somewhat higher than normally used indicated a predominance of x-x type dimers with greatly diminished amounts of x-y type dimers (results not shown). In some patterns, weak spots appeared below the diagonal with the fully reduced mobilities of x-type subunits, but with first-dimension mobilities about midway between those of the main group of dimers and the monomers (see Fig. 2). These spots apparently originated from x-x type dimers with more compact structures than those of the main group and appeared to be formed selectively from only one type of x subunit, perhaps as a consequence of the two subunits being linked together by disulfide bonds at both ends as opposed to linkage at one end. An exact fit resulting from a combination of only one type of subunit might explain the selectivity.

Ratio of HMW-GS Dimers to Monomers Produced by Partial Reduction

The ratio of HMW-GS dimers to HMW-GS monomers in the supernatant solution after the partial reduction of residue glutenin was dependent on the volume of the buffer used for the reduction. In general, as the volume increased, the overall protein concentration and the relative amounts of dimers of HMW-GS decreased, perhaps because of the increase in the absolute amount of DTT available for reduction or because of a mass action effect. The ratio of HMW-GS dimers to HMW-GS monomers was plotted as a function of the protein concentration of the supernatant solution after partial reduction for both wheat varieties (Fig. 5). Figure 5 indicates that the amount of dimers increased as a function of protein concentration in the supernatant solution and that Yecora Rojo yielded a higher ratio of dimers to monomers than did Anza at any given protein concentration.

Analysis of Nullisomic-Tetrasomic Lines

Two-dimensional analysis (SDS-PAGE, unreduced vs. reduced) of the mixture of n-t lines (results not shown) did not show any obvious recombinations of x and y subunit dimers. Spots corresponding to the 2-12 dimers and to the 7-8 dimers were clearly present, but not the possible recombinations of subunits 1Dx2 and 1By8 or of subunits 1Bx7 and 1Dy12.

DISCUSSION

Effects of Partial and Total Reduction on y-Type HMW-GS

The y-type HMW-GS liberated by partial reduction contained at least one intramolecular disulfide bond per monomer, as indicated by their faster mobilities in SDS-PAGE in comparison with those of their fully reduced forms. These intramolecular disulfide bonds apparently held the molecules in a more compact conformation than was characteristic of the fully reduced species; hence they had faster mobilities. The greater mobility for partially reduced subunit 10 than for partially reduced subunit 12 indicates a more compact form for subunit 10. This is surprising insofar as these two subunits are 95% similar in their amino acid sequences and identical through their N-terminal domains, which includes five of the seven cysteines (Thompson et al 1985, Anderson et al 1989, Greene et al 1988). The remaining two cysteines are located near the C-terminus of the protein molecule, one at the end of the domain corresponding to repeating sequences and the other in the C-terminal domain. There are some small sequence differences in this region between the two C-terminal cysteines that are likely to be responsible for the anomalous SDS-PAGE mobility of the fully reduced forms of 1Dy10 and 1Dy12 (Flavell et al 1989, Goldsbrough et al 1989). It is possible that the two C-terminal region cysteines might form an intramolecular disulfide bond (Shani et al 1992), with sequence differences in the region of these cysteines resulting in significantly different conformations of the polypeptide chains (assuming that the N-terminal and C-terminal domains do not form intramolecular disulfide linkages), but we did not see any evidence for this.

Although our results do not allow us to conclude with certainty that the fast-moving forms of the y-type subunits resulted from the retention of one or more native disulfide bonds, we have seen nothing to indicate a conversion to these fast-moving forms with time during our partial reductions. Even in experiments where only 15 min of reduction took place, the y-type subunits were present only as fast-moving forms. Mobility differences for partially reduced forms of HMW-GS 1Dy10 and 1Dy12 relative to the fully reduced forms have been reported (Lawrence and Payne 1983, Goldsbrough et al 1989), but they appear to be considerably smaller than those we observed.

Intramolecular Disulfide Bonds in x-Type Subunits

The appearance of three close bands for subunit 1Dx5 in one-dimensional gels (Fig. 1) suggests that there is likely to be at

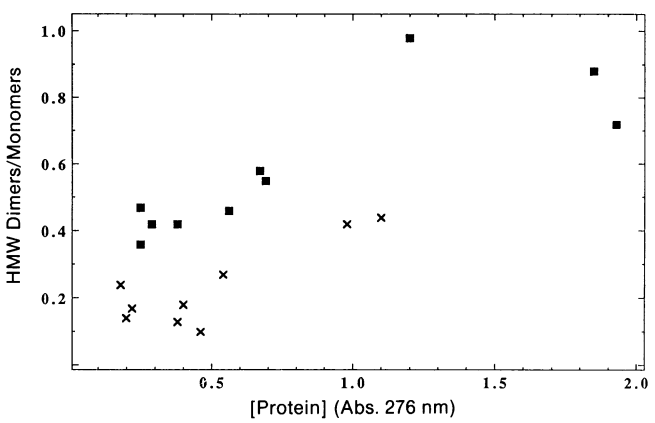

Fig. 5. Plot of dimer formation versus protein concentration. The ratio of high-molecular-weight (HMW) dimers to HMW monomers obtained by densitometry of two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns was used as measure of the amount of dimers present in partially reduced residue glutenin. This ratio was plotted as a function of the protein concentration as represented by the absorbance at 276 nm for extracts of partially reduced glutenin from Yecora rojo (■) and Anza (×) residues.

least one intramolecular disulfide bond present in the partially reduced form of this particular subunit and that several isoforms result from partial reduction. These isoforms presumably result from interactions of the four cysteine residues found near the N-terminus of the protein rather than from interactions between the N-terminal cysteines and the single C-terminal cysteine. Folding of the protein in such a way as to allow the single cysteine near the C-terminus to form a bond with the cysteines of the N-terminal region seems unlikely since such a major folding of the presumably rodlike HMW-GS (Shewry et al 1989) would be likely to produce an even greater mobility difference than we observed. Subunit 1Dx5 is unusual among x-type HMW-GS in having four cysteine residues near the N-terminus in contrast to the usual three (Greene et al 1988), with the fourth situated near the beginning of the repeat region. A possible interpretation of our result is that in native glutenin each polypeptide chain of 1Dx5 has one intramolecular disulfide that results from linkage of any one of the three usual N-terminal cysteine residues with the unusual fourth residue, the remaining cysteines participating in intermolecular cross-linkages. Normal x-types must form one N-terminal domain intramolecular cross-link or none. In the former case, there would be one N-terminal domain and one C-terminal domain intermolecular cross-linkage, whereas in the latter case, three N-terminal region intermolecular cross-linkages would result in branching occurring at the N-terminal domain of this subunit. In the special case of subunit 1Dx5, because of the extra cysteine residue, two or four of the cysteines near the N-terminal end of the molecule must be involved in intermolecular cross-linking.

The fast-moving forms of all x-type subunits that we observed, usually as minor components along with predominantly forms of normal mobility, must have at least one intramolecular disulfide remaining from the native form or, alternatively, produced during the partial reduction process through refolding of the polypeptide chain and reoxidation of cysteine residues to form an intramolecular linkage. The observation that these fast-moving forms were enhanced in a vigorously stirred solution supports the possibility that the faster-moving forms of x-type subunits are not representative of native structures, but more information will be necessary before this can be concluded with confidence.

Dimers of HMW-GS

Dimers of HMW-GS have been observed previously after the partial reduction of glutenin (Lawrence and Payne 1983; Graveland et al 1985), along with evidence for these dimers being formed primarily between x-type and y-type subunits (Lawrence and Payne 1983). In the work described here, a more extensive set of dimers was produced, and dimers combining two x-subunits were sometimes noted. The failure to observe x-x dimers consistently might result from their being either more or less stable than x-y dimers under the conditions we studied. Some preliminary evidence indicated that they were favored at higher levels of reducing agent than were optimum for producing the x-y dimers.

It is possible that the dimers we observed after the partial reduction of residue glutenin resulted from the reoxidation of HMW-GS monomers extracted into the supernatant solution and so may not represent arrangements of subunits in native glutenin. The possibility that an equilibrium exists in which dimers might be formed as well as dissociated was examined through an experiment in which flour of two different lines of Chinese Spring, one line having only subunits 1Dx2 and 1Dy12 and the other having only subunits 1Bx7 and 1By8, were mixed and then partially reduced in the same way as normal flours. Because Chinese Spring does not express a chromosome 1A-coded HMW-GS, the individual aneuploid lines form dimers composed of 1B-coded x-y pairs (1Bx7 and 1By8) or 1D-coded x-y pairs (1Dx2 and 1Dy12) of subunits. If the dimers we have observed during partial reduction formed as a result of reoxidation of HMW-GS monomers, then the mixture of the two aneuploid lines should have yielded dimers consisting of a 1B-1D subunit pair in addition to dimers consisting of only 1B or only 1D subunits. We found no evidence for such recombination, which supports our conclusion that the dimers observed for Yecora Rojo represent native structures and are not artifacts of the experimental procedure. From the results shown in Figure 5, it appears that the good-quality wheat (Yecora Rojo) had a relatively greater ratio of HMW-GS dimers to HMW-GS monomers produced by partial reduction of the residue fraction than did the poor-quality wheat (Anza). The stability of x-y type pairings is likely to be more resistant to reduction in good-quality wheats.

We have provided evidence through partial reduction and SDS-PAGE analysis of residue glutenin that all HMW-GS can form intramolecular disulfide bonds and have suggested that some of these bonds (particularly those of the y-type HMW-GS and of x-type subunit 5) are likely to be characteristic of the subunits as they exist in native glutenin. Furthermore, we have demonstrated that partial reduction of residue glutenin produces significant amounts of dimers composed of x-y pairs linked by an intermolecular disulfide bond. We also provide evidence that these dimers reflect arrangements of HMW-GS in the native glutenin polymers. In addition to x-y dimers, we have found dimers of two identical x-type subunits with mobilities close to those of the x-y type dimers. The selectivity might indicate that they are formed from partial reduction of the more compact x-x dimers mentioned above in the Results section.

Whether the dimers of HMW-GS are arranged in longer strings of HMW-GS in the native residue glutenin to form a backbone from which strings of LMW-GS branch, as proposed by Graveland et al (1985), or whether HMW-GS exist mainly as short strings, as would be expected from a random polymerization model (Kasarda 1989), remains to be determined. In any case, on the basis of our present work, it seems likely that x-y dimers are a significant structural element in residue glutenin. In consideration of the good correlation of residue glutenin with quality factors, such as loaf volume (Moonen et al 1982), the x-y type structures may play a role in determining quality differences. Some recent results (Shani et al 1992) obtained by reoxidation of reduced HMW-GS (expressed in bacteria) are partly in accord with our findings, particularly their finding of a stronger tendency for x-type subunits to form oligomers in comparison with y-type subunits. In addition, Gao et al (1992) have found similar x-y and x-x dimers resulting from partial reduction of doughs. This suggests that the stability of these dimers is sufficient to survive dough mixing. Also in agreement with our findings, Gao et al (1992) did not find evidence of y-y dimers, although Köhler et al (1992) reported finding disulfide linkages between the two immediately adjacent N-terminal cysteines of y-type subunits and their equivalents in another y-type subunit through enzymatic degradation of gluten. We cannot explain this apparent discrepancy at present. Perhaps, the y-y interactions of the type described by Köhler et al (1991) represent only a small proportion of the interactions of HMW-GS subunits with one another.

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