A Recombinant Protein of Two High Molecular Weight Glutenins Alters Gluten Polymer Formation in Transgenic Wheat*

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Wheat high molecular weight glutenin subunits (HMW-GS) are the most important determinants of its superior quality for making leavened bread. Following synthesis, these proteins are sequestered into the endoplasmic reticulum and assemble into extremely large elastic polymers, linked by noncovalent and intermolecular disulfide bonds. To study the structural requirements for the assembly of HMW-GS, we have expressed in transgenic wheat a recombinant protein between two cognate x- and y-type subunits. In contrast to the natural polymerized x- and y-type HMW-GS, a significant amount of the recombinant subunit remained monomeric. Nonreducing SDS-polyacrylamide gel electrophoresis, coupled with limited proteolysis, showed that the monomeric form of the recombinant subunit contained an unusual intramolecular disulfide bond, linking an N-terminal cysteine to the single C-terminal cysteine residue. In addition, sucrose gradient analysis revealed that this intramolecular disulfide bond impeded the ability of the recombinant subunit to assemble into polymers. Despite of its altered assembly, a notable amount of the overexpressed recombinant subunit was also present in glutenin polymers. Moreover, its presence significantly altered the subunit composition of the polymer. Our results show that it is possible to modify gluten assembly and properties by expressing recombinant HMW-GS in transgenic wheat, and have a major implication for the improvement of wheat breadmaking quality.

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Six multiallelic HMW-GS genes have been identified, two at each of the homoeologous Glu-I loci located on group 1 chromosomes (4). The HMW-GS genes at each Glu-I locus encode two closely related subunit types, termed x and y on the basis of their mobility in SDS-PAGE (4). The x- and y-type genes appear to have been formed by a duplication of a single ancestral gene early during wheat evolution (4). They have been maintained separately as two distinct genes in each HMW-GS locus, rendering x- and y-types of different wheat genomes more homologous to each other than to x- and y-types encoded by the same genome (3). This is in contrast to other groups of wheat storage proteins, which comprise multigene families and apparently evolved by unequal crossing over (5, 6).

The x- and y-type HMW-GS share a very similar structure (about 80% amino acid sequence similarity): a large central domain composed of repeating amino acid sequences rich in glutamine and proline, flanked by N- and C-terminal domains made up of nonrepetitive sequences that contain highly conserved cysteine residues (Fig. 1A) (3, 7). Nevertheless, x- and y-type subunits differ in several important features. x-types usually have three cysteines in the N-terminal domain, while y-types have five; both types have one cysteine in the C-terminal domain, but y-types have an additional cysteine within the repeat region in close proximity to the C-terminal domain. In addition, there are some differences between the two types in the composition and order of repeats within the central domain (3). Both x- and y-type HMW-GS exhibit retarded mobility in SDS-PAGE, suggesting that they possess a specific structural conformation that is not entirely denatured in SDS. Physical studies (3, 8–10), as well as scanning tunneling microscopy (11), have indicated that HMW-GS may possess a rodlike shape. It has been suggested (3) that this is a consequence of the repetitive domain adopting a b-spiral structure, similar to that formed by certain repeating sequences in elastin. However, such a structure was supported neither by computer modeling (12) nor by temperature-dependent water absorption studies (13).

Despite extensive studies (see Ref. 1, and references therein), the role of HMW-GS structure in polymer assembly within the ER is largely not understood. As x- and y-types have been maintained separately in evolution, we hypothesized that the differences between them may play an important role in their...
ability to interact. We previously produced several recombinant constructs between x- and y-types and expressed them in bacteria (14). The recombinant subunits showed unexpected SDS-PAGE mobilities, suggesting that despite the close sequence similarity between x- and y-types, they may have substantial conformational differences, particularly in their N-terminal domains (14). In the present report, we have analyzed the assembly of one of these recombinant HMW-GS and its incorporation into polymers in the native environment of wheat endosperm. The recombinant subunit was found to possess a modified assembly pattern, and its expression also altered glutenin polymer formation in the transgenic plants.

**EXPERIMENTAL PROCEDURES**

**Transgenic Wheat Lines**—Grains were obtained from hexaploid bread wheat cultivar Bobwhite, transgenic lines 1 and 4, expressing relatively low and high levels of the recombinant subunit, respectively, and from a line stably transformed only with the selectable marker plasmid, as a control (15).

**Sequential Extraction of Proteins from Mature Grains**—Grains were ground with a mortar and pestle in a buffer containing 0.1 M NaCl, 0.1 mM Tris-HCl (pH 8.0), 1 mM EDTA and then centrifuged at room temperature for 15 min at 13,000 × g. The resulting supernatant, containing the salt-soluble proteins, was kept. For further analysis and the pellet resuspended in a buffer containing 2% SDS, 0.1 mM Tris-HCl (pH 8.0), 1 mM EDTA. Following extraction for 2 h at room temperature with agitation, the suspension was centrifuged as described above and the supernatant was collected and termed “SDS-soluble,” kept. The pellet was then re-extracted as above, with the inclusion of 1% β-ME. Following centrifugation, the supernatant was collected and termed “SDS-insoluble.”

For partial reduction, the salt-soluble or SDS-soluble supernatant was treated with various concentrations of DTT for 30 min at room temperature.

For quantification of the proportion of monomeric recombinant HMW-GS, seeds from the “high expressing” transgenic line were homogenized with 100 mM DTT, and both halves were centrifuged to obtain either reduced and oxidized forms of the recombinant subunit, which migrates faster than the endogenous migrates in SDS, while the larger ones are insoluble unless intermolecular disulfide bonds are first broken by reducing agents (20).

**SDS-PAGE and Immunoblot Analysis**—Protein samples were dissolved in SDS sample buffer either lacking or containing 5% β-ME as described in the text and fractionated by SDS-PAGE (16). The separated gels contained 10% (w/v) acrylamide and 0.05% (w/v) bisacrylamide. Proteins were stained with Coomassie Blue as described previously (17). In some cases, the stained protein bands were quantitated using an image analysis software system (MacBAS). For immunological detection, the proteins were transferred from the gels to a nitrocellulose membrane and reacted with a mixture of anti-x- and anti-y-type HMW-GS sera (at a dilution of 1:3000 each), kindly provided by P. R. Shewry (University of Bristol, Long Ashton, United Kingdom) and R. Rubin (Rehovot, Israel). Immunoreacting bands were detected using ECL detection reagents (Amersham).

Two-dimensional SDS-PAGE—SDS-soluble supernatants were partially reduced with 1 mM DTT, dissolved in sample buffer lacking β-ME and subjected to SDS-PAGE. The designated lane was excised from the gel, equilibrated in upper Tris buffer (0.125 mM Tris-HCl (pH 6.8), 0.1% SDS) containing 5% β-ME for 15 min at room temperature, laid on the right halfway down the stacking gel, and subjected to electrophoresis. Electrophoresis was then continued as usual.

Following electrophoresis, sample lanes were subjected to a second dimensional separation on reducing SDS-PAGE as described above. For N-terminal amino acid sequence analysis, in-gel digested samples were electroblotted onto polyvinylidene difluoride membranes and stained with Coomassie Blue (19). The two major digestion products of ~60 and ~50 kDa were excised and subjected to protein sequence analysis in an Applied Biosystems sequencer (model 477A).

**RESULTS**

**Association of Recombinant Dy10-Dx5 HMW-GS with Glutelin Polymer**—To unravel the role of x- and y-type HMW-GS in polymer assembly, we have transformed wheat with a gene encoding a recombinant D-genome x-type subunit (Dx5), in which the N-terminal domain was substituted with the homologous domain from the y-type subunit of the same genome (Dy10) (Fig. 1A). The N-terminal domains of these subunit types possess 80% amino acid sequence similarity (Fig. 1B). The transformation and expression of the recombinant subunit in endosperm tissue of transgenic wheat has been previously described in detail (15). Wheat HMW-GS form aqueous buffer-insoluble polymers of various sizes. The smaller ones are soluble in SDS, while the larger ones are insoluble unless intermolecular disulfide bonds are first broken by reducing agents (20).

Mature grains from transgenic lines 1 and 4, expressing relatively low and high levels of the recombinant subunit, which migrates faster than the endogenous Dx5 subunit (Fig. 2, A and B, lanes a–c), was also present in SDS-soluble and SDS-insoluble fractions. As shown in Fig. 2 (A and B, lanes a–c), wild type HMW-GS subunits A2*, Dx5, Bx7, By9, and Dy10 (15) of the control wheat were all present in complexes that partitioned between the SDS-soluble and SDS-insoluble fractions. In the two transgenic genotypes, the recombinant subunit, which migrates faster than the endogenous Dx5 subunit (Fig. 2, A and B, lanes e, f, h, and i; see arrow on the right), was also present in SDS-soluble and SDS-insoluble polymers. However, a significant amount was also present in the salt-soluble fraction in both transgenic genotypes (Fig. 2, A and B, lanes d and g). Notably, in the high expressing transgenic line, a larger proportion of the endogenous subunits was present as SDS-soluble, smaller polymers compared with non-transformed control plants (Fig. 2, A and B, cf. lanes b and c versus lanes h and i). This phenomenon was much less pronounced in the “low expressing” transgenic line.

The Salt-soluble Recombinant Subunit Exists as a Monomer—As shown in Fig. 3A (lane a), in the absence of a reducing agent, the salt-soluble recombinant subunit migrated in SDS-PAGE as two bands within the range of ~80–110 kDa, one with the expected size of a reduced monomer, and the second migrating slightly faster. No glutenin oligomers were detected in this lane. Glutenin subunits containing intramolecular disulfide bonds generally migrate in nonreducing SDS-PAGE slightly faster than the reduced ones (14, 21). Thus, to test whether the rapidly migrating band corresponded to a monomeric form of the recombinant subunit that contained an intramolecular disulfide bond, the salt-soluble fraction was treated with increasing concentrations of DTT before SDS-PAGE. As shown in Fig. 3A (lanes b–e), upon addition of this reducing agent, the rapidly migrating band was gradually converted into a form that migrates at the position of the regularly migrating reduced monomer.

Although the salt-soluble recombinant subunit was not linked to other polypeptides by intermolecular disulfide bonds,
it could still be present in complexes linked by noncovalent interactions that were dissociated in the SDS gel. To test this possibility, the salt-soluble fraction was separated on a 5–20% sucrose gradient in the absence of any denaturing agent. As this gradient separates proteins up to around 150 kDa in size, monomeric HMW-GS of around 90 kDa are expected to migrate to the middle of the gradient, while dimers and oligomers are expected to sediment to the bottom. As shown in Fig. 3B, the salt-soluble recombinant subunit sedimented to the middle of the gradient, between the 67- and 150-kDa markers, corresponding to the expected size of a monomer.

The Novel Intramolecular Disulfide Bond in the Recombinant Subunit Interferes with Its Ability to Participate in Intramolecular Noncovalent Interactions—As HMW-GS assemble by both intramolecular disulfide bonds and noncovalent interactions, we wished to test whether the intramolecular disulfide bond within the recombinant subunit affected the latter’s ability to interact noncovalently. To address this, the salt-soluble extract from the transgenic plant was divided into two parts. One was fractionated on a similar gradient supplemented with 100 mM DTT. As shown in Fig. 4, while the oxidized form of the recombinant subunit mostly sedimented as a monomer (Fig. 4, panel A), the reduced subunit was partially assembled into homooligomers that sedimented to the bottom of the gradient (Fig. 4, panel B). These oligomers were apparently associated by noncovalent interactions.

Mapping the Intramolecular Disulfide Bonds in the Recombinant Subunit—Although HMW-GS contain several lysine residues, a previous study showed that only one, located in the central repetitive region, is exposed to cleavage by endoprotease Lys-C (22) (see Fig. 1). Thus, treatment of x- or y-type HMW-GS with this endoprotease results in the cleavage of the intact subunits into two relatively large fragments, containing either the N- or C-terminal regions (22). We therefore used limited Lys-C proteolysis to test whether the intramolecular disulfide bond in the recombinant subunit was formed between two cysteines in the N-terminal region or between an N-terminal and the single C-terminal cysteine. To address this, the salt-soluble extract was fractionated either in the absence or presence of reducing agents to generate the rapidly migrating oxidized monomer and slowly migrating reduced monomer, respectively. These two bands were excised separately and subjected to limited, in-gel proteolysis with Lys-C, as described under “Experimental Procedures.” As shown in Fig. 5A (lanes a and b), upon limited proteolysis of the reduced monomeric form of the recombinant subunit from the salt-soluble fraction with Lys-C, the intact subunit (migrating in the SDS gel with a molecular mass of ~110 kDa) was partially cleaved into two fragments of ~60 and ~50 kDa. Based on the unique position of the lysine residue in the central part of this subunit (Fig. 1A, Dy10-Dx5), the ~60- and ~50-kDa polypeptides apparently correspond to the N- and C-terminal regions of the recombinant subunit, respectively. This was confirmed by N-terminal sequencing of the first 6 amino acids of the two resulting peptides. The ~60-kDa band yielded Glu-Gly-Glu-Ala-Ser-Arg, and the ~50-kDa band yielded Gly-Gln-Gln-Pro-Gly-Gln. These sequences revealed a complete match to the N-terminal sequence of the mature Dy10 and the internal sequence start-
FIG. 2. Association of the chimeric subunit with the glutenin polymer. Seeds from a control line not expressing the recombinant subunit (a–c) and from the transgenic lines, expressing either low (d–f) or high (g–i) levels of the recombinant subunit, were sequentially extracted with a salt-containing buffer (a, d, and g), SDS buffer (b, e, and h), and SDS buffer containing β-ME (c, f, and i). Equal portions from each of the supernatants were fractionated in SDS-PAGE and either stained with Coomassie Blue (A) or subjected to immunoblot analysis (B) using anti-HMW-GS sera. The location of the recombinant subunit is marked by an arrow on the right. The wild type subunits are identified on the right of panel A. Molecular weight (MW) markers are indicated on the left.

When the oxidized band of the recombinant subunit was treated with Lys-C and refractionated in SDS-PAGE in the absence of reducing agents, a significant proportion of the rapidly migrating band (corresponding to the normal location of the oxidized form) was converted into a slower migrating form that migrated near the reduced monomeric form (Fig. 5A, lane d). Such a significant conversion was not observed in a control that was not treated with Lys-C (Fig. 5A, lane c). We thus hypothesized that the newly formed, slower migrating oxidized monomeric band of the recombinant subunit (Fig. 5A, lane d, upper band), which was produced upon Lys-C treatment, represented the fraction of the oxidized recombinant subunit that was actually cleaved at the centrally located Lys-C sensitive site, but that the two fragments were still held together by a disulfide bond naturally formed between the N- and C-terminal regions. This digestion has apparently altered the conformation of the oxidized protein into a more extended, slower migrating form. To test this possibility, both the rapidly and slowly migrating monomeric bands from lane d of Fig. 5A were excised from the gel, and the proteins were then eluted from the bands and refractionated on reducing SDS-PAGE. As shown in Fig. 5A (lanes e and f), the slower migrating band was separated into the two expected ~60- and ~60-kDa polypeptides, while the rapidly migrating band was not, supporting our hypothesis. Our hypothesis that the oxidized monomeric form of the recombinant monomeric subunit contained an intramolecular disulfide bond between the N- and C-terminal region was also confirmed by an additional experiment. Similar lanes to those shown in Fig. 5A (lanes c and d), were separated in a second dimensional reducing gel (Fig. 5, panels B and C). The band exhibiting the slower migration (double-headed arrow on top of Fig. 5C) was separated into the ~60- and ~50-kDa polypeptides in the Lys-C-treated sample (indicated by arrows on the left of the two spots in Fig. 5C). No such polypeptides were detected at this location (arrow on top of Fig. 5B) in the untreated sample.

Interaction of the Recombinant Subunit with Endogenous HMW-GS and Its Assembly into Polymers—Although a significant portion of the recombinant subunit was present as monomer, the fact that it was also present in SDS-soluble and SDS-insoluble fractions indicated that it was also partially capable of interacting with itself or with other endogenous HMW-GS. To address this point, we first analyzed the nature of the recombinant subunit in the SDS-soluble fraction by

FIG. 3. Characterization of the salt-soluble chimeric subunit. A, the salt-soluble fraction from transgenic seeds expressing high levels of the recombinant subunit was treated with increasing concentrations of DTT, fractionated on SDS-PAGE, immunoblotted, and reacted with anti-HMW-GS sera. The locations of the reduced monomer (Red.) and the more rapidly migrating monomeric band containing an intramolecular disulfide bond (Ox.) are indicated. Molecular weight (MW) marker positions are indicated to the right. B, the same salt-soluble extract as in panel A was fractionated on a 5–20% sucrose gradient. Individual fractions were separated on SDS-PAGE under reducing conditions and immunoblotted with anti-HMW-GS sera. The sedimentation positions of molecular weight markers along the gradient are shown on top of the panel, while the position of the recombinant subunit (arrow) and the migration of molecular weight (MW) markers in the SDS-PAGE are indicated on the left.
An intramolecular disulfide bond ties the C-terminal cysteine to one of the N-terminal cysteines in the recombinant subunit. A, the rapidly migrating reduced bands and the slowly migrating oxidized bands of the monomeric recombinant subunit were excised from a gel similar to the one shown in Fig. 3A, and fractionated on nonreducing SDS-PAGE (lanes a and b and lanes c and d for the reduced and oxidized monomers, respectively). Fractionation was performed with (lanes b and d, labeled + on top of the lane) or without (lanes a and c, labeled − on top of the lane) in-gel supplementation of endoproteinase Lys-C. The upper and lower bands of lane d were each excised, eluted from the gel, and fractionated on reducing SDS-PAGE (lanes e and f, respectively). The location of molecular weight (MW) markers is indicated on the left. The location of the reduced (Red.) and oxidized (Oxi.) monomeric forms of the recombinant subunit is indicated by arrows on the left. The two proteolytic fragments, corresponding to ~60 and ~50 kDa, are indicated by arrows on the right. B and C lanes from undigested and Lys-C-digested samples, similar to those shown at lanes c and d of panel A, were separated on a second dimension gel in the presence of excess β-ME (panels B and C, respectively). The location in the first dimensional gels, of the oxidized monomer (Oxi.), the reduced monomer (arrow in B), and the Lys-C cleaved oxidized monomer (double-headed arrow in C) are indicated on the top of the panels. The migration of the reduced monomeric form of the recombinant subunit (arrow on the right) and the two proteolytic fragments (arrows on the left in panel C) are shown for the second dimensional separation. The location of molecular weight (MW) markers is shown in between panels B and C.

SDS-PAGE separation in the absence of reducing agent. In control seeds, HMW-GS from this fraction were exclusively present in polymers that were stacked at the top of the gel (Fig. 6, lane a, arrow). However, in transformed seeds, a significant amount of the SDS-soluble recombinant subunit appeared as reduced and oxidized monomers (Fig. 6, lane b). To test whether some recombinant subunit from this fraction was also present in oligomers or polymers, together with the natural HMW-GS, the top part of lane b was excised, treated with excess β-ME, and fractionated in a second SDS-PAGE under reducing conditions. As shown in Fig. 6 (lane c), this treatment liberated a substantial amount of recombinant subunit, as well as the natural subunits, implying that they were all linked by disulfide bonds into polymers.

Next, we wished to identify the specific intermolecular interactions of the recombinant subunit. The SDS-soluble fraction was partially reduced with 1 mM DTT (which generally liberates HMW-GS monomers as well as oligomers linked by intermolecular disulfide bonds; Ref. 21), and subjected to two-dimensional SDS-PAGE, the first dimension in the absence of β-ME and the second in an excess of β-ME. As shown in the top horizontal first dimensional gel in Fig. 7A, treatment of the SDS-soluble fraction from control seeds with 1 mM DTT liberated several bands: HMW-GS monomers (M), two major bands of HMW-GS dimers (D), and two additional faint bands that apparently represented HMW-GS trimers (T). Upon fractionation on the second dimension reducing SDS-PAGE, the HMW-GS monomers migrated in an expected diagonal, while the oligomers were dissociated into monomers that appeared to the left of the diagonal. The pattern of appearance of the spots on the left of the diagonal suggested that they were liberated from various x-x and x-y type homodimers and heterodimers (21). HMW-GS dimers of y-y types were not detected, in agreement with previous reports in which y-y dimers were not found upon partial reduction of HMW-GS (21, 24).

Addition of 1 mM DTT to the SDS-soluble fraction from the transgenic seeds liberated four dimer and four trimer HMW-GS bands (Fig. 7B, top horizontal first dimensional gel). Each of these groups of dimers and trimers included the two dimer and trimer bands present in nontransformed seeds (compare with Fig. 7A), as well as two additional, more slowly migrating bands (additional dimers are shown by upward pointing arrows), which apparently represent new oligomers containing the recombinant subunit. To analyze this further, the first dimensional gel at top of Fig. 7B was separated on a second dimensional SDS-PAGE in the presence of excess β-ME. This fractionation revealed two major spots to the left of the diagonal (single-headed arrows), which were not present in the gel from the control plant (Fig. 7, compare A and B). According to their migration, these two major spots were apparently liberated from homodimers and homotrimers of the recombinant subunit. A closer look at the second dimensional panel of Fig. 7B revealed several new spots, which, according to their migration, were apparently liberated from various heterodimers containing the recombinant subunit and other endogenous x- and y-type subunits. One of these spots of the B-genome x-type subunit Bx7 is indicated by an arrowhead. This subunit was apparently liberated from a heterodimer also containing the recombinant subunit. A major spot that deviated to the right of the diagonal appeared only in transgenic seeds (Fig. 7B, double-headed arrow) and apparently represented the oxidized monomeric form of the recombinant subunit.

The pattern of assembly of the recombinant subunit into large polymers was also studied by analyzing the pattern by which individual subunits from the SDS-insoluble fraction were rendered SDS-soluble upon addition of increasing concentrations of DTT. As shown in Fig. 8, the pattern of liberation of subunits into the SDS-soluble fraction was similar when com-
paring complexes from transgenic and control plants, and both the wild type and recombinant subunits exhibited a similar release pattern. Notwithstanding, in the transgenic seeds, the largest glutenin complexes (that were dissociated only by the highest concentration of a reducing agent) still contained appreciable amounts of the overexpressed recombinant subunit (Fig. 8, lane h). This suggests that the portion of recombinant subunit that was incorporated into polymers, assembled in a manner similar to the endogenous subunits.

Quantitation of the Proportion of the Monomeric Recombinant HMW-GS—The proportion of the recombinant subunit present as monomers in the high expressing transgenic line was determined in the following experiment. Seeds were homogenized in an aqueous buffer containing 2% SDS (see “Experimental Procedures”), and the homogenate was divided into two. One half was supplemented with 100 mM DTT. The homogenates were then centrifuged to obtain either SDS-soluble (monomers and oligomers) or total HMW-GS (in their reduced monomeric form) supernatants. The two supernatants were then fractionated on 5–20% sucrose gradients. As shown in Fig. 9A, the SDS-soluble recombinant subunit partitioned between monomers that sedimented in the middle of the gradient (fractions 6–8), and a small proportion of oligomers that sedimented to the bottom of the gradient together with the endogenous HMW-GS (fraction P). The total reduced recombinant subunit also sedimented as expected to the middle of the gradient together with the endogenous HMW-GS (Fig. 9B, fractions 6–8). Fractions 6–8 from each of the gradients shown in panels A and B were pooled and subjected to four equal sequential 2-fold dilutions. Equal volumes from each dilution were then fractionated on reducing SDS-PAGE (Fig. 9C), and the bands corresponding to the recombinant subunit (labeled by an arrow on the right) were densitometrically quantitated (see “Experimental Procedures”). This analysis revealed that the monomeric form (M) of the recombinant subunit amounted to about 50–60% of the total (T) recombinant subunit (Fig. 9C, cf. lanes 1, 3, 5, and 7 with lanes 2, 4, 6, and 8, respectively).

**DISCUSSION**

The Recombinant HMW-GS Exhibits an Altered Assembly Pattern—To unravel the mechanism of HMW-GS assembly, we have expressed in the endosperm tissue of transgenic wheat, a recombinant protein containing the N-terminal region of a y-type subunit, and the central repetitive plus the C-terminal regions of an x-type subunit. Assembly of the recombinant subunit was studied by several different approaches: (i) partition between salt-soluble, SDS-soluble, and SDS-insoluble fractions; (ii) fractionation of HMW-GS on sucrose gradients; and (iii) identification of HMW-GS oligomers released from larger polymers upon treatments with low concentrations of a reducing agent (25). Two lines of evidence have suggested that assembly of the recombinant subunit into polymers was altered. (i) In contrast to the wild type subunits, which were entirely present in SDS-soluble and SDS-insoluble polymers, a significant portion of the recombinant subunit was present in the salt-soluble fraction; and (ii) sucrose gradients and SDS-PAGE in the absence of reducing agents directly showed that, unlike wild type subunits, which were present entirely in polymers, a significant amount (~50–60% in the high expressing line) of the recombinant subunit was monomeric.
assembly with other subunits was due solely to its excess molar ratio, the entire excess would have expected to be found as homo-oligomers.

The Monomeric Subunit Contains an Intramolecular Disulfide Bond between an N- and a C-terminal Cysteine—A large proportion of the monomeric Dy10-Dx5 subunit migrated in SDS-PAGE more rapidly than the expected migration of the reduced monomer, suggesting that it contained an intramolecular disulfide bond. This was confirmed by the disappearance of this rapidly migrating band upon addition of DTT. Furthermore, limited proteolysis with endoproteinase Lys-C, in the presence or absence of reducing agents, followed by N-terminal amino acid sequence analysis of the proteolytic fragments, showed that the intramolecular disulfide bond was formed between the single C-terminal cysteine of this recombinant subunit and one of the N-terminal cysteines. Association of N- and C-terminal cysteines by an intramolecular disulfide bond is not in accord with the suggested rodlike structure of HMW-GS (3, 6, 26), in which these cysteines would be too distant from each other to interact. Whether the apparent proximity of the N- and C-terminal regions shown here represents an altered structural conformation of the recombinant subunit or reflects an unexplored feature of the general structure of HMW-GS is an exciting area of research for future studies.

Previous studies showed that intermolecular disulfide bonds between N-terminal cysteines of y-type subunits and the C-terminal cysteine of x-type subunits are rather abundant in glutenin polymers (20, 22). This suggests that the N- and C-terminal domains of x- and y-type HMW-GS possess particular structural conformations that encourage the formation of intermolecular disulfide bonds between x- and y-type subunits. The special conformation of N-terminal domains of x- and y-type subunits was also previously inferred from expression of complementary recombinant x-y and y-x subunits in bacteria (14). Thus, based on our previous and present observations, we suggest that the intramolecular disulfide bond formed within the recombinant subunit is due to the installation, on the same polypeptide, of a y-type N-terminal domain and a x-type C-terminal domain, which naturally form intermolecular disulfide bonds between them.

Incorporation of the Recombinant HMW-GS Alters the Composition of the Glutenin Polymers—While over half the recombinant subunit was monomeric, the remainder was assembled into polymers of both small and large sizes, which partitioned between the SDS-soluble and SDS-insoluble fractions. Moreover, the gradual liberation of HMW-GS from the SDS-insoluble fraction upon progressive reduction also implied that upon assembly, the recombinant subunit was highly competent for incorporation into even very large polymers. This incorporation rendered the overexpressed recombinant subunit the most abundant subunit in these large polymers (Fig. 8) and measurably altered their structure and composition. At the same time, overproduction of the recombinant subunit reduced the extent of incorporation of wild type subunit into large (SDS-insoluble) polymers (Fig. 2). The molecular basis for this observation has still to be elucidated.

A Hypothesis for the Control of Assembly of HMW-GS within the ER—Our results suggest that during folding of the HMW-GS within the ER, the cysteines in their N- and C-terminal domains are accessible for two different maturation steps that compete with each other: (i) formation of intermolecular disulfide bonds with cysteines from other subunits and incorporation into glutenin polymers and (ii) formation of intramolecular disulfide bonds that may interfere with their polymerization. However, the natural HMW-GS have evolved in

![Figure 9](image-url)
such a way to significantly favor the formation of inter- versus intramolecular disulfide bonds. This preference may have been reversed by the cysteine configuration in the recombinant subunit. The structural requirements for favoring the formation of intermolecular disulfide bonds between HMW-GS are not understood. However, based on our results, a possible explanation is that this phenomenon is related to some evolutionary-derived conformational differences between the N- and C-terminal domains of x- and y-type subunits. Indeed, the N-terminal domain of Dy10 contains an insertion of 18 amino acids, including two adjacent cysteines not present in Dx5, while the C-terminal regions of the two subunit types possess some critical changes in a few amino acids, e.g. three scattered proline residues in Dy10 versus one serine and two leucine residues in Dx5 (23). Further modifications of these residues are required to dissect their significance in HMW-GS assembly.

Implications for Improving Bread-making Quality of Transgenic Wheat—Our results show unequivocally that it is possible to change the composition of the glutenin polymer by expression of modified HMW-GS in transgenic wheat. Moreover, we have shown that modification of the polymers may not only be achieved by altering the nature of a recombinant protein, but also by controlling the level of its expression. Our laboratories continue to use this approach to both understand the mechanism of modified HMW-GS in transgenic wheat and improve wheat bread-making quality.

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