Mechanisms of Assembly of Wheat High Molecular Weight Glutenins Inferred from Expression of Wild-type and Mutant Subunits in Transgenic Tobacco*

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Following sequestration into the endoplasmic reticulum, wheat high molecular weight glutenin subunits (HMW-GS) assemble into polymers through intermolecular disulfide bond formation. These polymers, which also include low molecular weight glutenin subunits (LMW-GS), have a broad distribution of molecular mass reaching up to several million daltons. To study the mechanism of assembly of the HMW-GS, we have expressed x- and y-type HMW-GS in transgenic tobacco plants. Both types, when expressed individually or in combination, were incorporated into polymers. Partial reduction of polymers formed by different subunits resulted in different patterns of release of homodimers, heterodimers, and monomers. This suggested different arrangements of intermolecular disulfide bonds or different peptide conformations in the vicinity of the disulfide bonds linking x-x, y-y, and x-y type HMW-GS. A mutant of the x-type subunit, lacking a conserved cysteine in the C-terminal domain, assembled into oligomers linked by intermolecular disulfide bonds, but not into large polymers. This mutant was deposited, however, in dense protein bodies, similar to those formed by the native HMW-GS, suggesting that polymer formation and packaging into protein bodies may be the result of different types of interactions. Pulse-chase labeling of proteins in wheat endosperm showed that the assembly of the HMW-GS into insoluble polymers occurs by a slow process which apparently continues after the initiation of protein body formation.

Wheat seed storage proteins are synthesized on membrane bound polysomes, sequestered into the endoplasmic reticulum (ER), and then accumulate in dense protein bodies (PB) (1). These storage proteins, also known as gluten proteins, are generally classified into two groups, gliadins and the glutenins. Gliadins are monomeric proteins soluble in alcohol-water solutions, while the glutenins are largely insoluble in alcohol-water solutions and consist of two types of subunits: the high- and low-molecular weight glutenin subunits (HMW-GS and LMW-

GS) (1, 2). Glutenins possess a unique characteristic in that, following sequestration into the ER they assemble into polymers linked by intermolecular disulfide bonds, having a molecular mass ranging from about 80 kDa up to at least several million daltons (2, 3). These polymers account for the elastic properties of wheat flour doughs. Although solvents containing SDS or acetic acid dissolve a considerable proportion of these glutenin polymers, some, presumably the largest ones, are insoluble even in SDS solution unless reducing agents that break disulfide bonds are present in the extraction medium (4–7).

In bread wheat (genome AABBDD), six multiallelic HMW-GS genes have been identified, two at each of the homeologous loci Glu-1 located on group 1 chromosomes (8, 9). Alleles of each of the six HMW-GS genes have been cloned and sequenced (10–14). The HMW-GS encoded at each Glu-1 locus form two general classes which were originally termed x- and y-types on the basis of mobility in SDS-PAGE (15). The respective genes are thought to have diverged from a common progenitor (12).

All HMW-GS genes encode proteins with three domains: a large central domain composed of repeating sequences and the N- and C-terminal domains made up of nonrepetitive sequences that contain all or most of the cysteine residues (Fig. 1A) (16). Although x- and y-types share a similar structure, they differ in several important features. Whereas the x-types usually have 3 cysteines in the N-terminal domain, the y-types have 5 cysteines in this domain. Both x- and y-type subunits have 1 cysteine in the C-terminal domain, but the y-types have an additional cysteine, located 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 (16). Physical studies (4, 16–18) have indicated that HMW-GS are rod-like in shape and it has been suggested that this is a consequence of the repetitive domain adopting a β-spiral structure, similar to that formed by certain repeating sequences in elastin (16, 17).

Several theories have been proposed concerning the arrangements of the various glutenin subunits in glutenin polymers. Ewart (3, 4, 19, 20) provided evidence for the presence of a largely linear arrangement of glutenin subunits in the glutenin polymers, but did not rule out the possibility of some branching.

Gravelaard and associates (5) hypothesized that glutenin polymers are built of large strings of HMW-GS, assuming preferential assembly of these subunits within the ER. Kasarda (2) hypothesized that the different HMW-GS and LMW-GS are randomly arranged in the polymers and noted that a random polymerization process would not favor long strings of HMW-GS inasmuch as the LMW-GS predominate in glutenin on a molar basis.

Recent studies in several laboratories showed that partial reduction of glutenin polymers resulted in the liberation of dimers and possibly trimers of HMW-GS (21–22), suggesting at
least some degree of association between HMW-GS in the gluten polymers. Notably, dimers composed of x-y type subunits were more abundant, compared to the x-x combinations, whereas y-y combinations were not found (21, 22). However, whether this ratio between dimers resulted from preferential interactions between x- and y-type subunits or from their preferential liberation from the glutenin polymer has not been determined.

Studies on the mechanism of assembly of HMW-GS and their packaging into PB in wheat endosperm cells generally suffer from the large heterogeneity of subunits in these aggregates. This problem may be overcome by expressing in transgenic tobacco plants chimeric genes encoding single and specific combinations of wild-type and mutant HMW-GS (23). In the present report we show that both x- and y-type subunits could assemble either individually or in combination to form large, SDS-insoluble polymers. Moreover, the various polymers exhibited slightly different patterns of breakdown into oligomers upon reduction. We also found that the single cysteine on the C-terminal domain of the x-type HMW-GS was important for its assembly into the large polymers, but not for its assembly into small oligomers or for its packaging into PB.

EXPERIMENTAL PROCEDURES

Construction of Chimeric HMW-GS Genes—For construction of the chimeric 35S-Dx2 gene, an ApoI to NsiI DNA fragment, containing the coding DNA sequence of Dx2 (kindly provided by Dr. D. Soll; Ref. 13) plus 151 nucleotides upstream of the AUG and 35 nucleotides downstream of the stop codon, was blunt ended with mung bean nuclease and cloned into the Smal site of pUC-19. Bal3 was then used to eliminate most of the 5'-nontranslated DNA, leaving 39 nucleotides upstream of the AUG. A Smal I to NsiI DNA fragment was then excised from the pUC-Dx2 plasmid and cloned into the same sites in pJD30 (kindly provided by Dr. R. Gallie), between a 5' 3'S promoter plus 131 nucleotides and a 3'-polyadenylation signal of the nopaline synthase genes. For construction of the chimeric 35S-Dy12 gene, a StuI to NsiI DNA containing the coding DNA sequence of Dy12 (kindly provided by Dr. R. Thompson; Ref. 14), was used to replace the analogous StuI to NsiI DNA fragment of Dx2 in the chimeric 35S-Dx2 gene.

Construction of the Mutant Dx2 Lacking the Cysteine in the C-terminal Domain—Site-directed mutagenesis was used to change the TCC codon of the cysteine on the C-terminal domain of Dx2 into a TCC serine codon. Two genomic DNA fragments coding for the C-terminal part of Dx2 were cloned into a two fragment plasmid: (i) an NcoI to HindIII fragment was cloned into plasmid pET-8c (25) which contains an NcoI cloning site adjacent to a 3' polyadenylation signal of the Escherichia coli lacZ gene. (ii) The HindIII to HindIII DNA fragment of Dx2 was cloned into a Bluescript KS plasmid (Stratagene, La Jolla, CA) so that the initiation codon of the HMW-GS gene is adjacent to the HindIII site. The plasmid is then reextracted from the PET-8c in a buffer containing 20 mM Tris (pH 7.6), 50 mM KCl, 10 mM MgCl2, 0.3 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 10% (v/v) sucrose. The homogenate was filtered through four layers of cheesecloth and layered on a continuous 10-50% (v/v) metrizamide gradient (30). Centrifugation was performed as described previously (31). Aliquots (200 μl) each fraction were diluted 1:5 with the above buffer and centrifuged for 30 min at 28,000 × g, 4 °C. The pellets were extracted with 70% ethanol and processed for SDS-PAGE analysis as described.

SDS-PAGE and Western Blot Analysis—Proteins were dissolved in SDS sample buffer lacking or containing β-ME at concentrations indicated in the text, and then fractionated on 7.5% (w/v) or 10% (w/v) gels by SDS-PAGE as previously described (32). Staining of the proteins with Coomassie Brilliant Blue was as previously described (33). For Western blot analysis the proteins were transferred to a nitrocellulose filter (0.2 μm) and immunoblotted with anti-x or y-type HMW-GS sera, kindly provided by Dr. P. R. Shewry and R. Rubín, respectively. Detection of proteins on the immunoblot was performed using the ECL detection reagents (Amersham).

Pulse and Pulse-Chase Labeling of Developing Wheat Grains—For studying the incorporation of newly synthesized HMW-GS into polymers, developing wheat grains at 17 days after anthesis were cut into thin slices and incubated for 20 min in [35S]methionine (10 μCi/ml, Du Pont New England Nuclear) at room temperature. The slices were then washed with water, ground in SDS sample buffer containing or lacking 1% β-ME, and the labeled subunits were fractionated by SDS-PAGE and detected by fluorography.

RESULTS

Expression of the HMW-GS Dx2 and Dy12 in Transgenic Tobacco Plants—To study the processes of assembly of wheat HMW-GS and their packaging into PB, we have transformed tobacco plants with chimeric genes encoding the wheat HMW-GS Dx2 (x-type) and Dy12 (y-type) under the 35S-promoter of cauliflower mosaic virus (Fig. 1B). To test for the expression of the HMW-GS, proteins were extracted from leaves of several transgenic plants in 70% ethanol containing 1% β-ME and fractionated in SDS-PAGE. Extracts from the various transgenic plants, transformed with the Dx2 and Dy12 genes, exhibited an additional Coomassie Brilliant Blue-stained protein band that co-migrated with the proteins from the control nontransformed plants (Fig. 2). These bands were absent in the control nontransformed plants. The identity of these proteins as HMW-GS was confirmed by their cross-reactivity with anti-HMW-GS sera in Western blots (data not shown). Representative SDS-PAGE patterns from plants 2-5 (Dx2) and 12-22 (Dy12), which were chosen for further analyses, and are shown in Fig. 2, lanes c and e, respectively. Plants 2-5 and 12-22, were crossed to yield T1 progenies which co-expressed the Dx2 and Dy12 HMW-GS (Fig. 2d).

Assembly of the HMW-GS in Leaves of Transgenic Plants—In
wheat endosperm, the HMW-GS associate with each other and with other storage proteins to form complexes, linked primarily by disulfide bonds (3). The largest complexes are SDS-insoluble (5) and can be rendered SDS-soluble upon treatment with reducing agents (2). These characteristics of the glutenin polymers in the representative common wheat line, Deganit, are demonstrated in Fig. 3A. When a mature grain from this cultivar is extracted with SDS in the absence of reducing agent, approximately 40% of the HMW-GS are found in SDS-insoluble complexes (Fig. 3A, lanes a and b). In contrast, upon extraction with SDS and β-ME, more than 95% of the HMW-GS become SDS-soluble (Fig. 3A, lanes c and d). We have adopted this characteristic as a measure for the assembly of the HMW-GS in leaves of the transgenic tobacco plants. Subunits Dx2 and Dy12, expressed in transgenic plants either individually or in combination, assembled into large complexes that were essentially (90% or more) insoluble in SDS in the absence of reducing agents (Fig. 3B, lanes b–g). Addition of a reducing agent to the extraction medium rendered these HMW-GS almost entirely SDS-soluble (Fig. 3B, lanes h–m), indicating that the SDS insolvability stemmed primarily from associations by intermolecular disulfide bonds, similarly to the HMW-GS in wheat en-

Fig. 1. A, a general schematic diagram of the x- and y-type HMW-GS. N, N-terminal regions; arrows, central repetitive regions; C, C-terminal regions; S, location of cysteine residues. The cysteine on the C-terminal domain of Dx2 is circled. B, a schematic diagram of the chimeric genes utilized in the present study. 35S, the 35S promoter; O, the DNA coding for the ω mRNA leader sequence (24); HMW-GS, the coding DNA sequences of the HMW-GS genes; TER, the nopaline synthase 3’-polyadenylation signal; pGA492, binary Ti plasmid (25).

Fig. 2. Expression of the HMW-GS in leaves of transgenic tobacco plants. Proteins were extracted in 70% alcohol and 1% p-ME, fractionated in SDS-PAGE and detected by Coomassie Blue staining. CS, the wheat cultivar Chinese Spring used as a reference; Control, nontransformed tobacco; 2-5, a transgenic plant expressing both Dx2 and Dy12 HMW-GS genes; 12-22, a transgenic plant expressing the HMW-GS Dy12 gene. The position of the natural Dx2 and Dy12 subunits is shown on the left.

Fig. 3. SDS solubility of HMW-GS polymers. SDS-soluble (S) and insoluble (I) HMW-GS were extracted as described under “Experimental Procedures,” fractionated in SDS-PAGE, and detected by Coomassie Blue staining. For clarity, only the HMW-GS are presented. A, HMW-GS extracted from mature grains of the wheat cultivar Deganit in the absence (lanes a and b) and in the presence of β-ME (lanes c and d). B, HMW-GS extracted from leaves of transgenic tobacco plants expressing Dx2, Dy12, or Dx2 plus Dy12 genes, as indicated on top of each lane, in the absence (lanes b–g) or in the presence of β-ME (lanes h–m) in the first extraction buffer. CS, the cultivar Chinese Spring used as a reference. The position of the natural Dx2 and Dy12 subunits is shown on the left.

dosperm cells (Fig. 3A).

The nature of the HMW-GS polymers formed in the transgenic plants was studied further by analyzing the oligomers (mainly dimers) obtained following partial reduction. Initially, the SDS-insoluble fractions were re-extracted in SDS containing relatively low concentrations of β-ME (0.01 and 0.05%). However, this fraction was quite resistant to reduction (or to solubilization of partially reduced complexes) and no monomers or oligomers were released under these conditions (data not shown). Treatments with 0.1–1% β-ME caused the release of HMW-GS monomers, but essentially no oligomers (data not shown). Therefore, the partial reduction experiments were performed on the smaller HMW-GS complexes from the SDS-soluble fraction. In order to ascertain whether the released products were indeed related to HMW-GS, their detection was performed by Western blot analysis using anti-HMW-GS sera. As a reference we used the aneuploid line ditelosomic 1BS of the common wheat cultivar Chinese Spring, which lacks the long arm of chromosome 1B and thus possesses only the Dx2 and Dy12 HMW-GS. The subunit composition of the oligomers released from the glutenin polymers of this line was based on a previous report (21). When the soluble glutenin complex from this line was extracted and fractionated in the presence of 0.05% β-ME, the predominant product released, besides the monomeric subunits, was a heterodimer between subunits Dx2 and Dy12 (Fig. 4A, lane a, see single-headed arrow). Upon separation on a second dimension in SDS-PAGE containing 5%
concentrations shown on the top of each lane. The SDS-soluble polymers were treated with β-ME, at the concentrations shown on top of each lane, and the proteins were then fractionated in SDS-PAGE in the absence of reducing agents and detected in a Western blot with anti-HMW-GS serum. DT1BS, partial reduction of SDS-soluble glutenin polymers from the ditectosomic line 1BS of the cultivar Chinese Spring. B, storage proteins from the aneuploid line DT1BS were separated on two-dimensional SDS-PAGE as previously described (20) and detected by Coomassie Blue staining. The first dimension contained 0.5% β-ME, while the second dimension contained 5% β-ME. The single and double-headed arrows indicate the respective positions of the Dx2-Dy12 heterodimers and the Dx2 homodimers in part A, as well as their resulting monomers in the second dimension SDS-PAGE in part B. Dx2 and Dy12 represent the positions of the original monomers.

β-ME, this heterodimer was separated into the original Dx2 and Dy12 HMW-GS which were located outside the diagonal containing all of the original monomeric subunits from the first dimensional separation (Fig. 4B, bands marked by single-headed arrows). In addition, a fainter homodimer of Dx2, which migrates slightly slower than the heterodimer, was also detected. This homodimer of Dx2, which is marked by a double-headed arrow in Fig. 4B, was very faint in the reduction shown in Fig. 4A, lane a, and therefore it was not detected in the Western blot. Y-type dimers, which are expected to migrate slightly faster than the x-y dimers, were not detected in most experiments, while in some experiments they appeared as extremely faint bands.

When the Dx2 homopolymer from the tobacco plants was extracted and fractionated in the presence of 0.05% β-ME, a homodimer was clearly evident in addition to the monomer (Fig. 4A, lane b, see double-headed arrow). In contrast, no homodimer of Dy12 was detected under a similar partial reduction, despite the fact that Dy12 was assembled into large polymers (Fig. 4A, lane c). Extraction and fractionation of the HMW-GS complex from tobacco co-expressing Dx2 and Dy12 genes in the presence of 0.01% β-ME caused a preferential release of the Dx2-Dy12 heterodimer, while a fainter Dx2 homodimer was also evident (Fig. 4A, lane d). However, the relative intensity of the Dx2 homodimer was increased while that of the heterodimer was decreased upon extraction with higher concentrations of β-ME (Fig. 4A, lanes d-f). Also in this experiment, essentially no homodimer of Dy12 was released from the glutenin complex. Thus, despite of some differences in the extent of oligomerization and assembly into insoluble complexes compared to wheat endosperm cells, the above results show that the tobacco model system is suitable to assess the mechanism of HMW-GS assembly and the role of cysteine residues in the process.

**Assembly of a Mutant Dx2- Subunit Lacking the Cysteine in the C-terminal Domain**—It was suggested (5) that the HMW-GS may be linked end-to-end to each other through single disulfide linkages involving the cysteines in their N- and C-terminal domains (see Fig. 1A). Nevertheless, since there are several cysteines present in the N-terminal domains of each HMW-GS, more than one intermolecular disulfide bond could be formed by these cysteines. To address this issue, we expressed in transgenic tobacco plants a mutant of Dx2 in which the cysteine in the C-terminal domain was replaced by a serine residue (Dx2-), by using site-directed mutagenesis. First, we wished to study whether this Dx2- mutant could assemble into large SDS-insoluble polymers. Although this mutant subunit was expressed at a comparable level to that of the natural subunit, it was hardly incorporated into large complexes and was mostly present in the SDS-soluble fraction (Fig. 5). Nevertheless, this mutant was present in oligomers, linked through cysteines in the N-terminal ends, as evident from the Dx2- monomer and homodimer liberated upon partial reduction of the complex (Fig. 5, see double-headed arrow).

Although the analysis of the mutant Dx2- showed that HMW-GS can interact via intermolecular disulfide bonds between cysteines on the N-terminal domains, we wished to study whether assembly into large, SDS-insoluble complexes occurred predominantly by “head-to-tail” linkages between cysteines on the N- and C-terminal domains. To address this issue, we crossed tobacco plants expressing genes encoding the natural Dy12 and the mutant Dx2- subunits. If assembly into the large, SDS-insoluble complexes occurred predominantly by head-to-tail linkages, then the Dx2- would not be expected to become SDS-insoluble even when assembled in the presence of the natural Dy12 subunit. Moreover, by acting as a terminator during polymerization, the mutant Dx2- subunit should decrease the size of the Dy12 polymers. Alternatively, incorporation of the Dx2- subunit into the SDS-insoluble polymer might occur if Dx2- could readily form more than one intermolecular disulfide linkage by way of its 3 N-terminal cysteine residues.
As can be deduced from Fig. 7, the latter explanation appeared to best fit the results, as a large part of the Dx2- was incorporated into SDS-insoluble polymers when allowed to assemble in the presence of the natural Dy12 subunit. Partial reduction of the glutenin polymers formed by Dx2- and Dy12 caused the release of Dx2- homodimer and Dx2--Dy12 heterodimer, while only the Dx2- homodimer was obtained upon partial reduction of Dx2- homopolymer (Fig. 8, b-e), indicating that Dx2- definitely combined with Dy12 through its N-terminal domain.

Time Course of HMW-GS Assembly in Wheat Endosperm—
Previous studies from our laboratory had shown that packaging of wheat storage proteins into PB began shortly after their synthesis and that dense PB were already evident when allowed to assemble in the presence of the natural Dy12 subunit. Partial reduction of the glutenin polymers formed by Dx2- and Dy12 caused the release of Dx2- homodimer and Dx2--Dy12 heterodimer, while only the Dx2- homodimer was obtained upon partial reduction of Dx2- homopolymer (Fig. 8, b-e), indicating that Dx2- definitely combined with Dy12 through its N-terminal domain.

How the absence of this cysteine affects the packaging of Dx2- into PB. To address this issue, membrane homogenates from leaves of transgenic plants expressing the various HMW-GS genes, as well as from wheat endosperm as a control, were fractionated on a metrizamide density gradient. Both the wild-type Dx2 and Dy12 as well as the mutant Dx2-, when expressed in tobacco plants, were packaged into PB which sedimented down the gradient at a position similar to that of the natural PB from wheat endosperm (Fig. 11, A-D; see also Ref. 31).

DISCUSSION
Although it has been clearly established that the major interactions among the glutenins occur via intermolecular disulfide bonds (see Ref. 2 for review), thus far, the organization of subunits in glutenin complexes has been postulated mainly on the basis of chemical compositional analyses, sequence infor-
M, was added to one of them. Proteins were then fractionated in SDS-buffer lacking β-ME. The sample was divided into two parts and β-ME PAGE in the absence of reducing agents and detected by fluorography. HMW-GS is indicated on the developing wheat grains.

[35S]methionine for HMW-GS tenin complex by partial reduction (2, wheat glutenins, by expressing chimeric genes encoding indi-

We have taken a different approach to the study of the assembly of the HMW-GS in leaves based on the following reasons: (i) analysis of leaves is much more convenient and circumvents the long time needed to obtain seeds, and (ii) young leaves lack host storage proteins that might interact with the HMW-GS and interfere with the interpretation of results. Notably, the HMW-GS synthesized in tobacco leaves were incorporated more efficiently into SDS-insoluble polymers compared to the natural subunits from wheat endosperm. As folding and assembly of the HMW-GS takes place within the endomembrane system (1), it is possible that these differences were due to some variations in the microenvironment of the endomembrane system between tobacco leaves and wheat endosperm. Nevertheless, it is likely that folding and assembly of the HMW-GS were performed accurately in tobacco leaves as previous studies showed that the endomembrane system has been highly conserved during evolution and thus secretory proteins generally fold and assemble correctly when expressed in heterologous systems (37). Indeed, the HMW-GS produced in tobacco leaves migrated in SDS-PAGE to an identical position corresponding to natural subunits of the wheat endosperm.

Wheat HMW-GS migrate in SDS-PAGE slower than expected based on their actual Mr. This is likely to be due to specific conformations of β-turn and β-spiral that exist in the central repetitive region and are not completely denatured in SDS. Therefore, the identical migration in SDS-PAGE indicates that the subunits produced in tobacco leaves possess similar if not identical structures to those of the natural subunits. There are several other possibilities to explain the more efficient incorporation of the HMW-GSs in tobacco leaves, compared to wheat endosperm. 1) Polymers containing only HMW-GS may be less soluble than those containing both HMW-GS and LMW-GS, inasmuch as LMW-GS are smaller in size than HMW-GS and may also act as chain terminators (2). HMW-GS may be less soluble than those containing both HMW- and LMW-GS. 2) The solubility difference may reflect an intrinsic lower solubility of the HMW-GS compared to the LMW-GSs. 3) Long strings of HMW-GS may not be present in native glutenin complexes because they are broken up by LMW-GS interspersed at least occasionally in the strings. 4) The greater solubility of the natural glutenin complex may also be the consequence of branching of LMW-GS from the backbone chain (5), which may prevent the formation of strong secondary interactions between HMW-GS. At the present time, we cannot favor any of these possibilities. Yet, our results indicate that both x- and y-type subunits expressed individually readily form polymers containing only HMW-GS are expected to be larger on average, and hence less soluble than those containing both HMW- and LMW-GS. 2) The solubility difference may reflect an intrinsic lower solubility of the HMW-GS compared to the LMW-GSs. 3) Long strings of HMW-GS may not be present in native glutenin complexes because they are broken up by LMW-GS interspersed at least occasionally in the strings. 4) The greater solubility of the natural glutenin complex may also be the consequence of branching of LMW-GS from the backbone chain (5), which may prevent the formation of strong secondary interactions between HMW-GS. At the present time, we cannot favor any of these possibilities. Yet, our results indicate that both x- and y-type subunits expressed individually readily form long strings and that combined x-y expression is not a prerequisite for formation of large insoluble polymers.

Partial reduction of natural glutenin complexes from wheat endosperm produces a preferential release of x-y compared to x-x and y-y type dimers (21, 22). However, whether this is due to preferential assembly of x- and y-type subunits or differential conformation of different dimers is still unknown. In the present report we showed that homopolymers of x- or y-type...
subunits were apparently formed at a comparable efficiency to that of heteropolymers formed by x- and y-type subunits. Moreover, the relative amount of dimers released from the homo- or heteropolymers was similar to that released from natural glutenin complexes. Thus, although our study cannot rule out some preferential assembly of x- and y-type HMW-GS, it suggests that x-y heterodimers do not predominate in the glutenin complex, but are apparently released preferentially upon partial reduction. This is also supported by the observations that both in natural glutenin complexes (21, 22) and in the complexes of HMW-GS formed in tobacco leaves, the relative amount of x-x type dimers was increased with increasing the reducing agent concentration. The preferential release of x-y heterodimers from glutenin complexes may be determined by:

(i) specific conformational (steric) effects on the accessibility of the reducing agent to the disulfide bonds in the different subunits; and (ii) by differences in the number and locations of the cysteines involved in the formation of these bonds. As to the latter possibility, recent studies (36, 38, 39) have indicated that specific cysteines on the N- and C-terminal domains of the HMW-GS may form more than one type of disulfide bond. Although some disulfide bonds may be more prominent than others, these studies support our findings that argue against a higher level of specificity in the interactions between HMW-GS.

Our expression of the mutant Dx2-, lacking the cysteine on the C-terminal domain, showed that this cysteine was essential for the assembly of the HMW-GS into large SDS-insoluble homopolymers. This supports the likelihood that end-to-end polymerization of HMW-GS is an important contribution to the size of the polymers. However, our observation that the Dx2- mutant was able to assemble into small SDS-soluble oligomers, too large to enter a polyacrylamide gel (Fig. 5), showed that HMW-GS can also interact by "branch-type" intermolecular disulfide bonds between cysteines in their N-terminal domains. The presence of some branching within the glutenin polymers was supported by the finding that the mutant Dx2- was incorporated into SDS-insoluble polymers when it was co-expressed with the wild-type Dx12 subunit. The role of the y-type HMW-GS in glutenin assembly may be even more complex than that of x-type subunits as the former have 2 cysteines located near the C terminus (16). If both of these cysteines form intermolecular disulfide bonds, this would result in a higher degree of branching. Alternatively, the 2 cysteines on the C-terminal domain of the y-type subunits may be linked to one another by intramolecular disulfide bonds and thus may play a minor role in glutenin polymerization. We have recently shown that following in vitro assembly of y-type HMW-GS, expressed in bacteria, the cysteines in their C-terminal domain became linked by intramolecular disulfide bonds (38). Moreover, these bonds were relatively unstable and disappeared upon treatment with the sulfhydryl blocking agent iodoacetamide. Whether such intramolecular bonds also occur in vivo, or whether these cysteines may participate both in intra- and intermolecular disulfide bonds remains to be established.

Finally, the results of the present work indicate that the assembly of the HMW-GS into polymers and their packaging into PB involve different interactions. Moreover, assembly of the HMW-GS into polymers linked by intermolecular disulfide bonds may continue for a longer duration than that required for the initiation of PB formation (31). It is possible that the HMW-GS are packaged into PB as small polymers and then assemble into larger polymers through additional disulfide bond formation. Another explanation for the late occurrence of glutenin polymer insolubility might be greatly enhanced secondary interactions occurring during compression of PB and seed desiccation at the end of development.

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REFERENCES
1. Shewry, M. A., and Larkins, B. A. (1988) in The Biochemistry of Plants (Millin, B. J., ed.) pp. 297–345, Academic Press, New York.
2. Kasarda, D. D. (1989) in Wheat & Unique (Pernetz, Y., ed.) pp. 277–302, American Association Cereal Chemists, St. Paul, MN.
3. Ewart, J. A. D. (1987) J. Sci. Food Agric. 38, 277–289.
4. Ewart, J. A. D. (1988) Cereal Chem. 65, 65–100.
5. Halford, N. G., Förde, J. A. D., Greene, F. C., and Shewry, P. R. (1983) in Plant Proteins (Dausset, J., and Vaux, J., eds) pp. 205–219, Academic Press, New York.
6. Payne, P. I., Holt, L. M., Jackson, E. A., and Law, C. N. (1984) Philos. Trans. R. Soc. Lond. B Biol. Sci. 304, 359–371.
7. Lawrence, G. J., MacFetichie, P., and Weygand, C. W. (1988) Cereal Chem. 7, 109–112.
8. Anderson, O. D., and Greene, F. C. (1989) Theor. Appl. Genet. 77, 699–704.
9. Förde, J., Malpica, J. M., Halford, N. G., Shewry, P. R., Anderson, O. D., Greene, F. C., and Milis, B. (1988) Nucleic Acids Res. 16, 6817–6832.
10. Shewry, N. G., Förde, J. A. D., Greene, F. C., and Shewry, P. R. (1988) Theor. Appl. Genet. 77, 117–129.
11. Sugiyama, T., Raalski, A., Peterson, D., and Soll, D. (1985) Nucleic Acids Res. 13, 8729–8737.
12. Thompson, R. D., Bertels, D., and Harberd, N. P. (1985) Nucleic Acids Res. 13, 6863–6866.
13. Payne, P. I., Holt, I. M., and Law, C. N. (1981) Theor. Appl. Genet. 60, 229–236.
14. Shewry, P. R., Halford, N. G., and Tatham, A. S. (1989) Curr. Surf. Plant Mol. Cell Biol. 6, 163–219.
15. Tatham, J. A., Shewry, P. R., and Milis, B. J. (1984) FEBS Lett. 177, 205–208.
16. Matsuura, N., Nasano, G., Satoh, N., and Isu, Y. (1992) Biochem. Biophys. Res. Commun. 188, 1057–1064.
17. Ewart, J. A. D. (1966) J. Sci. Food Agric. 19, 617–623.
18. Ewart, J. A. D. (1970) J. Sci. Food Agric. 21, 402–402.
19. Lawrence, G. J., and Payne, P. I. (1983) J. Exp. Bot. 34, 254–267.
20. Wernert, W. E., Adelstein, A. E., and Kasarda, D. D. (1992) Cereal Chem. 69, 455–461.
21. Robert, L. S., Thompson, R. D., and Flavell, R. R. (1989) The Plant Cell 1, 569–578.
22. Galla, G., Lucas, W. J., and Walshe, V. (1989) The Plant Cell 1, 201–211.
23. Rosenberg, A. H., Lade, B. N., Chai, D., Lin, S., Dunn, J., and Studier, F. W. (1987) Gene (Amst.) 56, 125–135.
24. Hipuchi, R., Krummell, B., and Saki, K. (1988) Nucleic Acids Res. 16, 7251–7267.
25. An, G. (1986) Plant Physiol. 81, 86–91.
26. Hornsh, R. B., Steff, J. J., Hoffmann, N. L., Echelbrite, D., Rogers, S. G., and Prosp, R. T. (1986) Science 227, 1229–1321.
27. Deleted in proof.
28. Wallace, J. C., Galla, G., Kawata, E. C., Cuellar, R. E., Shewry, M. A., and Larkins, B. A. (1988) Science 240, 662–664.
29. Rubin, R., Levanyw, H., and Galla, G. (1992) Plant Physiol. 99, 716–724.
30. Lennart, U. K. (1970) Nature 217, 690–695.
31. Galla, G., and Feldman, M. (1981) Theor. Appl. Genet. 66, 77–86.
32. Millet, E., and Jennen, C. F. (1991) Physiol. Plant. 83, 591–596.
33. Geu, L., Ng, P. E. W., and Bashuk, W. (1992) Cereal Chem. 69, 453–455.
34. Tago, H. P., Adelstein, A. E., and Kasarda, D. D. (1992) Biochem. Biophys. Acta 1159, 13–21.
35. Colman, A., Bhansri, S., and Vallee, G. (1984) Biochem. Soc. Trans. 12, 932–937.
36. Shani, N., Stefen-Campbel, J., Adelstein, O. D., Greene, F. C., and Galla, G. (1992) Plant Physiol. 98, 433–441.
37. Köhler, P., Belitz, H. D., and Wieser, H. (1991) Z. Lebensm. Unters. Forsch. 192, 254–259.