Characterization of Low Molecular Weight Glutenin Subunits in Durum Wheat by Reversed-Phase High-Performance Liquid Chromatography and N-Terminal Sequencing

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ABSTRACT

Low molecular weight glutenin subunits (LMW-GS) from a residue preparation were fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC) of reduced subunits from two biotypes of the Italian durum wheat cultivar Lira that differ at the *Gli-B1/Glu*-B3* loci, such that Lira 42 has the LMW-1 pattern (associated with poor quality) and Lira 45 has the LMW-2 pattern (associated with good quality) of LMW-GS. N-terminal amino acid sequencing was conducted on the major protein fractions (peaks) that differed chromatographically between the two types. The fractions included four types of LMW-GS on the basis of their N-terminal sequences, which differed in their proportions among the various fractions. These N-terminal sequences corresponded to the Ser-His-Ile- (LMW-s) type, the Met-Glu-Thr- (LMW-m) type, the α-(gliadin) type, and the γ-(gliadin) type. The LMW-s type was strongly predominant in the Lira 45 preparation and predominant in the Lira 42 preparation. The LMW-m type was next most important in the Lira 45 preparations, but in the Lira 42 preparations, the percentage of α-type sequence was higher than the LMW-m type. Both α-type and γ-type sequences were stronger in Lira 42 than they were in Lira 45. Results indicated that differences in quality between the Lira biotypes may derive from: 1) the relative predominance in Lira 45 glutenins of the LMW-s type and the LMW-m type subunits, probably acting as glutenin polymer chain extenders; 2) the high proportion of the α-type and γ-type glutenin subunits in the Lira 42 glutenins, probably included in the glutenin polymers because they have an odd number of cysteines and act as glutenin polymer chain terminators; 3) the absolute amount of LMW glutenins, which is greater for LMW-2; or 4) some combination of these factors.

Wheat storage proteins determine many of the processing and quality characteristics of flour and semolina. Gliadins and glutenins are the two main groups of endosperm proteins. Gliadins have monomeric polypeptide chains, whereas glutenins form polymers of protein subunits linked by intermolecular disulphide bonds. Gliadins are coded by the complex loci *Gli-1* (γ- and ω-gliadins) and *Gli-2* (α- and β-gliadins), located on the short arms of group 1 and 6 chromosomes, respectively. Low molecular weight glutenin subunits (LMW-GS) are coded mainly by genes at the *Glu-3* loci, but some are coded by genes of the *Gli-1* and *Gli-2* loci (Lew et al 1992). The *Glu-1* loci, coding for high molecular weight glutenin subunits (HMW-GS), are present on the long arms of the group 1 chromosomes.

Durum wheat cultivars (AABB tetraploids) are grouped into two main types according to the allelic forms at the *Gli-B1/Glu-B3* loci. One presents γ-gliadin 42 and the associated LMW-GS of type 1 (LMW-1), and the other presents γ-gliadin 45 and the LMW-GS of type 2 (LMW-2) as first indicated by Payne et al (1984). Type 2 usually displays better pasta-making characteristics than does type 1. It is now commonly accepted that this is associated with the LMW-GS rather than with true γ-gliadins (Pogna et al 1988). Whether quality differences are due to the relative amounts of LMW-GS or to differences in the structure of particular allelic variants of these subunits is not known. Autran et al (1987) proposed a quantitative basis for quality differences in which good quality resulted from larger amounts of LMW-GS, although they did not exclude the involvement of physicochemical differences in LMW-GS.

To investigate the basis for quality differences in durum wheats, we analyzed the two biotypes of the Italian durum wheat cultivar Lira, one possessing the γ-gliadin 42 and LMW-1, and the other possessing the γ-gliadin 45 and LMW-2. These two biotypes differ in technological characteristics as expected, with the LMW-2 type having the better quality.

The aim of the present work was to determine whether there were structural differences between the LMW-GS corresponding to LMW-1 and LMW-2 that might also explain quality differences between these two types.

MATERIALS AND METHODS

Flours of the two biotypes of the durum wheat cultivar Lira (Lira 42 and Lira 45 representing poor and good quality, respectively) were kindly provided by Societá Produttori Sementi, Bologna, Italy. A mixture of reduced HMW-GS and LMW-GS was obtained according to the procedure of Singh et al (1991), with the exceptions that residue extraction was performed at room temperature and aklylation was omitted. Glutenin concentration was estimated spectrophotometrically with a protein assay reagent (Coomassie, Pierce, Rockford, IL), a system based on the Bradford (1976) method.

To detect possible quantitative differences in particular glutenin components between the two biotypes, the same amount of the glutenin protein mixture was injected in a reverse-phase high-performance liquid chromatography (RP-HPLC) system. Two different chromatographic systems were used, but results were comparable. The first system used a Vydac C_{18} semipreparative (10-mm $\times$ 25-cm) column (Vydac, Hesperia, CA), connected to a Spectra-Physics SP8700 solvent delivery system (Thermoseparations, San Jose, CA), an Isco V4 detector (Isco, Lincoln, NE), and an SP4270 integrator (Spectra-Physics). A linear gradient of 28–57% aqueous acetonitrile containing 0.05% trifluoroacetic acid in 55 min was used, with a flow rate of 1.5 ml/min. The second system used a Supelco C_8 column (4.6-mm $\times$ 25-cm) (Supelco, Inc., Bellefonte, PA) connected to Gold Beckman apparatus, version 3.11, comprising the solvent delivery module 126 and UV detector module 166 (Beckman, Palo Alto, CA). Two successive linear gradients of 29–43% aqueous acetonitrile in 50 min and 43–90% in 10 min (water and acetonitrile contained 0.07 and 0.05% trifluoroacetic acid, respectively) at a flow rate of 1 ml/min were used. In both cases, the columns were equili-

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brated at 50°C, and proteins were detected by UV absorbance at 210 nm.

Peaks, numbered sequentially, were collected separately and used for electrophoretic analysis and N-terminal sequencing. About 15 μg of fractionated proteins or 150 μg of total glutenin subunits were characterized by isoelectric focusing (IEF) versus sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), according to the technique described by Kasarda et al (1988), except for substitution of Triton-X for NP-40, and the use of 5% ampholytes (Pharmacia-LKB, Uppsala, Sweden) with the pH ranges 5–7 (1%), 7–9 (2%), and 8–10.5 (2%).

N-terminal amino acid sequence analyses were performed with a pulsed liquid phase amino acid sequencer (model 477A, Applied Biosystems, Foster City, CA) and an on-line phenylthiohydantoin (PTH)-amino acid analyzer (model 120A). Fractions from HPLC containing the proteins of interest were applied to a glass fiber filter pretreated with 3.0 μg of Biobrene Plus (Applied Biosystems). The results were analyzed by the methods described in Lew et al (1992), in which certain well-recovered (in the Edman degradation) amino acids at each cycle, such as methionine and isoleucine, that were characteristic of a sequence type, were emphasized in assigning the percentages of the various subunit types present in the fraction (Falk et al 1991). These percentages were combined with peak areas to calculate the overall percentages of the main sequence types in the glutenin fraction analyzed. Because alkylation of cysteine side chains was not performed (pyridylethylation is usually used in sequencing), PTH-cysteine residues, if present, were not detected. Cysteine is expected in the fifth position of the LMW-m type on the basis of DNA sequences (Cassidy and Dvorak 1991, D'Ovidio et al 1992a), although arginine and histidine also can occupy this position rather than cysteine in LMW-m (Lew et al 1992). Even after pyridylethylation, cysteine is poorly recovered (in the PTH form) during amino acid sequencing. This is also true of arginine and histidine. Consequently, the quantitation of any one of these three amino acids is usually not very good, and we felt that attempts to quantify the amounts of cysteine (or arginine and histidine) at cycle 5 were not justified in this preliminary work.

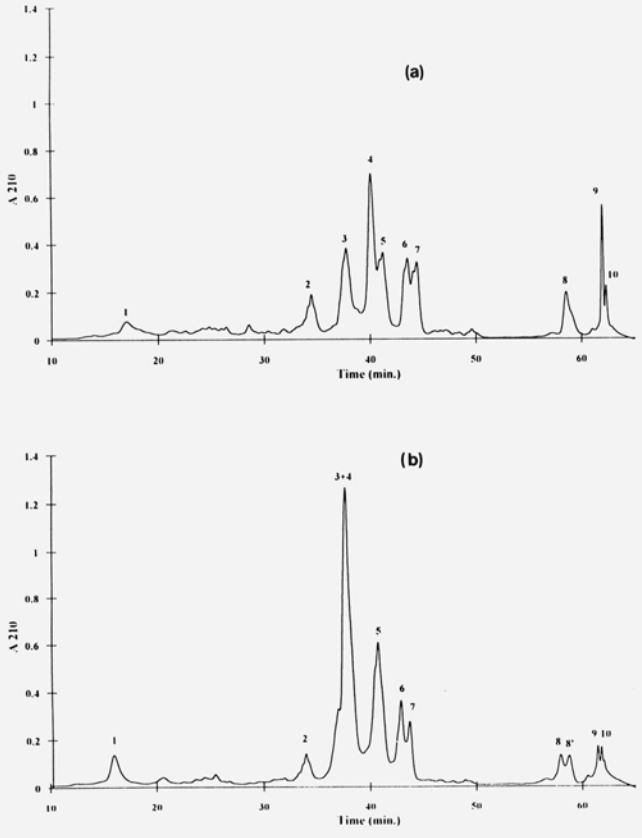

Fig. 1. Chromatographic separations of glutenin subunits of Lira 42 **(a)** and Lira 45 **(b)**.

RESULTS

The Lira 45 biotype yielded ~1.5 times as much protein in the residue prepared by the method of Singh et al (1991) as did the Lira 45 biotype. Chromatographic profiles of reduced glutenin subunits of Lira 42 and Lira 45 are shown in Figure 1. These chromatograms represent equal amounts of protein, as indicated by the Bradford assay, injected onto the RP-HPLC column. The two biotypes differed significantly one from the other in peaks 3, 4, 5, 8′, and 9. The two peaks designated as 4 differed for both retention time and area, while peaks 3, 5, and 9 differed only in area between the two biotypes. In Lira 45, peaks 3 and 4 usually coeluted, but a better separation could be obtained by rechromatographing the coeluting peaks with a shallower gradient (data not shown). Only a slight quantitative difference was found for peaks 1, 8, and 10. Peak 1 corresponded to the HMW-GS 20 and was greater in Lira 45, whereas peaks 8 and 10 were greater in Lira 42. The glutenin peaks that did not show more than very minor differences in quantity or retention time between the two biotypes (peaks 1, 2, 6, 7, 8, and 10) were not given major consideration on the assumption that they have little or no influence on quality characteristics. This assumption was supported by preliminary sequencing of the proteins corresponding to peaks 2 through 10, which did not indicate major differences in their compositions between the two biotypes.

One-dimensional SDS-PAGE patterns of fractions 1–10 of both biotypes are shown in Figure 2. These patterns indicated that

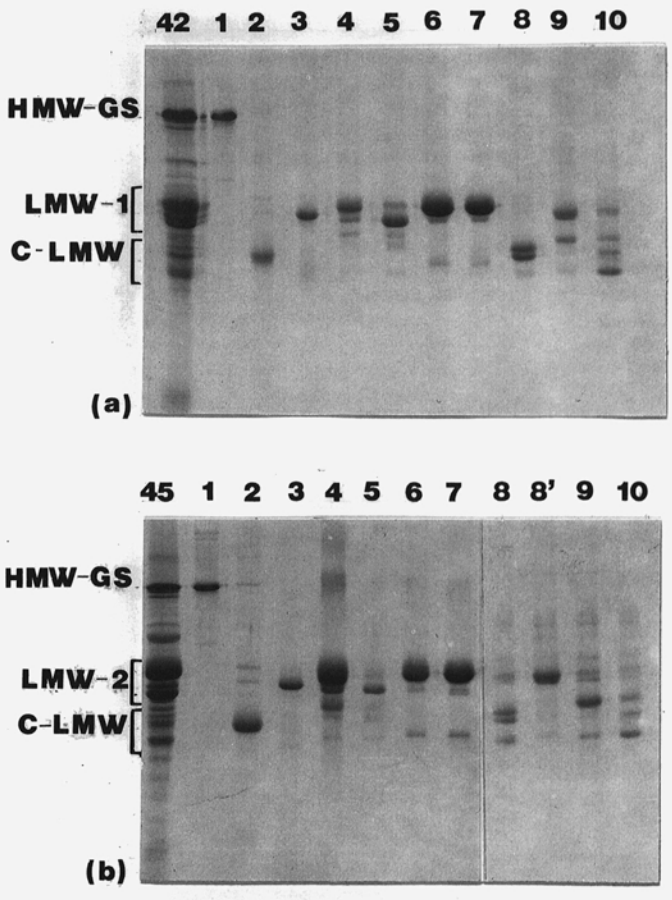

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fractions 1–10 of Lira 42 **(a)**, and Lira 45 **(b)**. Numbers on tracks correspond to the chromatographic fractions. First tracks correspond respectively to glutenins of Lira 42 and Lira 45 obtained according to the procedure of Singh et al (1991). HMW-GS = high molecular weight glutenin subunits; LMW-GS = low molecular weight glutenin subunits.

the predominant glutenin components were the classical LMW-1 and LMW-2 clusters that are included in the B mobility grouping (corresponding approximately in molecular weight from 40,000 to 50,000), but some C range subunits (molecular weights ranging from about 30,000 to 40,0000) were clearly present. Two-dimensional electrophoretic analysis was performed on the starting residue glutenin preparations (Fig. 3a, f) and fractions that differed between the biotypes (Fig. 3b–e and Fig. 3c–k). The spots most characteristic of a given fraction are indicated by the fraction number in the overall glutenin patterns of Fig. 3a, f. As can be seen in Figure 3 (a and f), the main difference between the LMW-1 and LMW-2 biotypes is in the relative position and intensity of the spots marked 4 in the patterns of each biotype. Spot 4 had a faster mobility in the second dimension in Lira 42 when compared to Lira 45, and it had more intense staining in Lira 45 than in Lira 42. This is also evident when comparing the 2D-electrophoretic patterns presented here with those reported by Morel and Autran (1990), relative to cultivars Kidur (LMW-GS type 1) and Agathe (LMW-GS type 2). Peak 3, the pattern shown in Figure 3g, was not completely pure due to the difficulty in separating peaks 3 and 4 in Lira 45 by RP-HPLC so that the more basic, slower moving spots in the pattern of Figure 3g actually result from contamination of the fraction by components mainly present in peak 4 (Fig. 3h).

Peaks 3, 4, 5, 8′, and 9 were analyzed also by N-terminal amino acid sequencing through 5–10 cycles. All early fractions showed multiple amino acids at each cycle, indicating that they were mixtures of proteins (LMW-s, LMW-m, and α-types), whereas the later fractions (peaks 8–10) yielded a sequence characteristic only of γ-type subunits. The percentages of each type in the individual peaks analyzed are given in Table I, and the overall percentages for the combined peak areas are given in Table II. These percentages must be considered only semiquantitative, however, because we did not apply any rigorous statistical analysis to the data. Additionally, there may have been some blocked proteins present in some fractions, as indicated by low yield and higher than expected background, although this was not likely to be a serious problem in the fractions we sequenced on the

TABLE I
Relative Percentages of N-Terminal Sequences Present in Fractions of Low Molecular Weight (LMW) Glutenin Subunits

Biotype	Sequence Type[a]	Fraction Number				
		3	4	5	8′	9
Lira 42	LMW-s	49	56	18	0	0
	LMW-m	26	15	39	0	0
	α	25	29	43	0	0
	γ_2	0	0	0	0	68
	γ_3	0	0	0	0	32
Lira 45	LMW-s	77	66	18	0	0
	LMW-m	14	17	63	0	0
	α	9	17	19	0	0
	γ_2	0	0	0	96	89
	γ_3	0	0	0	4	11

[a]LMW-s, S-H-I-P-G-L-; LMW-m, includes M-E-T-S-R-I-, M-E-T-S-H-I-, or M-E-T-S-C(?)-I-; α, V-R-V-P-V-P-; γ_2, N-M-Q-V-D-P-; γ_3, N-I-Q-V-D-P-. Single letter nomenclature for amino acids: C, cysteine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine.

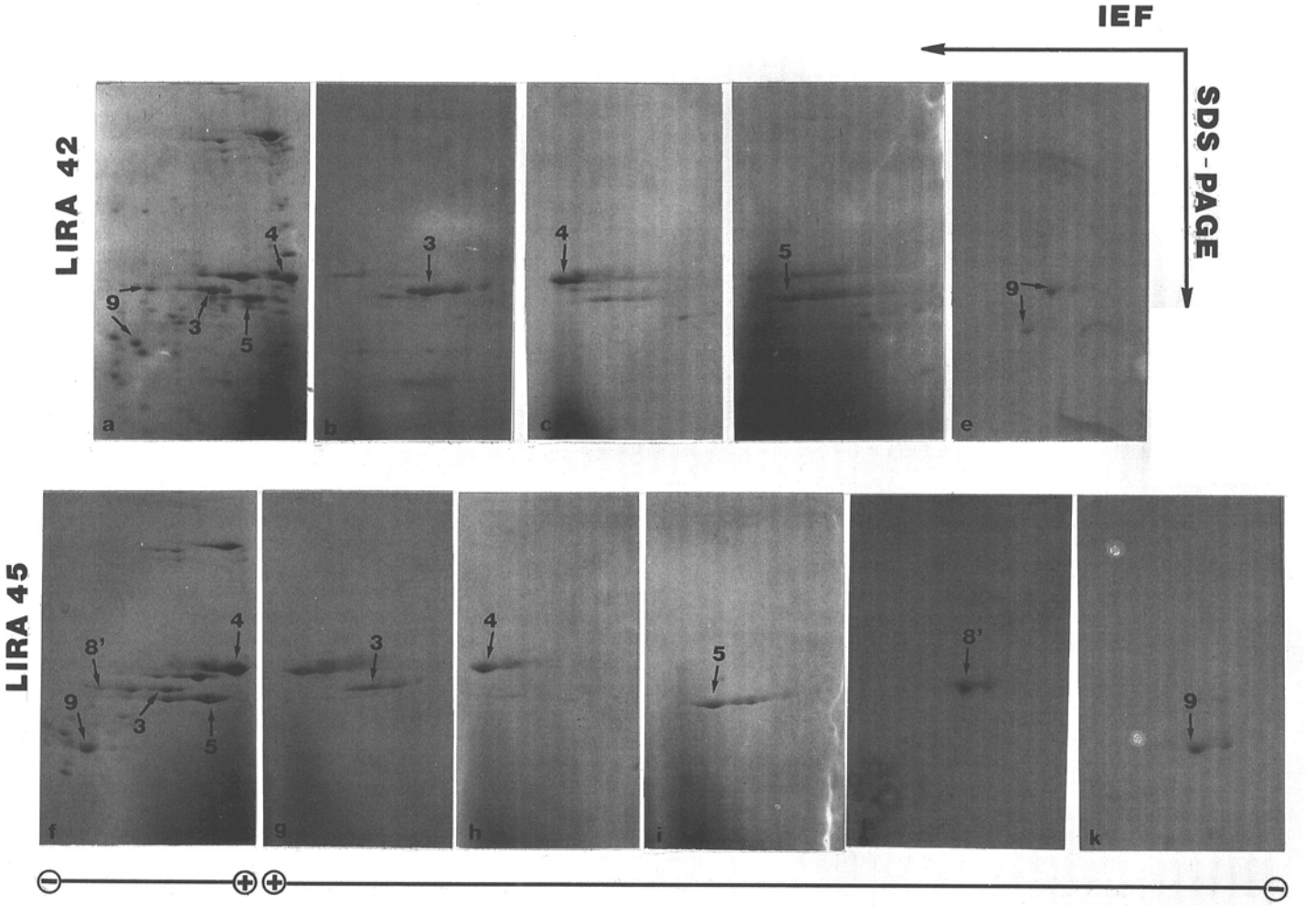

Fig. 3. Isoelectric focusing (IEF) vs. sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). a, Glutenin subunits of Lira 42. b–e, Peaks 3, 4, 5, 9 of Lira 42. f, Glutenin subunits of Lira 45. g–k, peaks 3, 4, 5, 8′, 9 of Lira 45. Electrophoretic spots have been numbered according to the corresponding chromatographic peaks.

basis of the actual versus expected sequencing recoveries.

The same four main types of sequences described by Tao and Kasarda (1989) and Lew et al (1992) for hexaploid wheats have been found in both biotypes of the durum wheat cultivar Lira. As also found for hexaploid wheats, LMW-s was clearly the predominant type of LMW-GS in both Lira 42 and Lira 45 (Tables I, II), making up a major proportion of the large peaks 3 and 4. The overall percentages of LMW-s on a mole percent basis in the starting residue glutenin preparations were 42 and 48% for the Lira 42 and Lira 45, respectively. The next most predominant subunit varied between the biotypes: α-type for Lira 42 (26%); the LMW-m type for Lira 45 (27%). Finally, for Lira 42, the LMW-m type and the γ-type subunits were present in approximately the same proportion (20 and 17%, respectively), whereas α-type subunits were predominant over γ-type subunits in Lira 45 (13 vs.7%).

The γ-gliadins were apparently a mixture of γ_2 and γ_3 types with a predominance of the sequence type that has methionine in the second position (γ_2) instead of isoleucine (γ_3) (Bietz et al 1977, Kasarda et al 1983). By comparing the electrophoretic patterns of fractions 8–10 (Figs. 2 and 3) it is possible to see that bands have the same mobilities in SDS-PAGE in the two biotypes, but that band 8′ corresponds in SDS-PAGE to a band present in peak 9 of Lira 42. This protein might have a mutation that does not affect molecular size and pI, but does affect the surface hydrophobicity slightly.

DISCUSSION

Triticum durum cv. Lira is particularly suitable for studying the mechanisms involved in quality differences because its two known biotypes differ only in the electrophoretic patterns of 1B-coded γ-gliadins, ω-gliadins, and LMW-GS (D'Ovidio et al 1992b), all of which are coded by clusters of closely linked genes on the short arm of chromsome 1B. Of particular interest is the presence of the group of LMW-GS designated LMW-1 in one biotype and the group designated LMW-2 in the other, which correlate with poor quality for the former and good quality for the latter. It is generally accepted that LMW-GS are primarily responsible for the determination of pasta-making properties (Payne et al 1984), and that the difference in quality between cultivars with LMW-GS type 1 and type 2 results mainly from a considerably greater amount of LMW-GS in the good quality type 2 cultivars (Autran et al 1987, Dachkevitch and Autran 1989).

Our results are in accord with the findings of Autran et al (1987) in that the insoluble glutenin fraction of Lira 45 (type 2) in our preparations was 1.5 times greater than that of Lira 42 (type 1). This presumably is an indication of more glutenin polymers in the higher molecular weight range for Lira 45 and, therefore, it is in agreement with Dachkevitch and Autran (1989), who found a positive correlation between quality characteristics and size of glutenin polymers. In addition however, we have found that this additional insoluble protein of the type 2 line consists largely of LMW-s type subunits although LMW-s was the predominant type in the glutenin of both lines. The LMW-m type was present in much lower amounts according to our sequencing-based analysis of proportions, but still contributed significantly to the glutenin of the good type, Lira 45 (Table II). On this basis, we suggest that it is likely that both LMW-s and LMW-m type subunits act mainly as chain extenders in glutenin polymerization (Kasarda 1989, Lew et al 1992). By chain extender, we mean that there are least two cysteine residues in the primary structure (sequence) of a subunit that tend to form intermolecular rather than intramolecular disulfide bonds.

TABLE II
Overall Percentages of the Main Sequence Types

	LMW-s[a]	LMW-m	α	γ
Lira 42	36	20	26	17
Lira 45	53	27	13	7

[a]Low molecular weight.

That LMW-s should be a chain extender seems somewhat surprising when its N-terminal sequence, which is all that is available for this type of subunit (Lew et al 1992), is compared with the sequence of an LMW-m type subunit from durum wheat, for which the complete sequence is available (based on the DNA sequence (Cassidy and Dvorak 1991, D'Ovidio et al 1992a). Lew et al (1992) have suggested that there are two likely cysteines that might be involved in intermolecular disulfide bond formation in an LMW-m type subunit: one is the cysteine at position 5, and the other at position 210 in the B11-33 sequence of Okita et al (1985). Support for the involvement of the latter cysteine (C^x) has been provided by Köhler et al (1993). Although there is some homology (similarity) between the LMW-s and LMW-m type sequences (Okita et al 1985), the LMW-s type of sequence had no cysteine at position 5, nor any through at least 30 cycles of sequencing (Lew et al 1992). If the two types of subunit are largely similar through the rest of the sequence, then LMW-s would be likely to have only one cysteine available for intermolecular disulfide bond formation (the one at position 210) and would thereby be a chain terminator, which would favor shorter glutenin polymers (lower molecular weight distribution).

We speculate that the apparent conflict would be resolved if it turned out that the C^{b*} cysteine described by Köhler et al (1993) as being present in LMW-GS and involved in intermolecular disulfide linkages were present in the LMW-s type subunit. This cysteine is likely to be found somewhere in the repeating sequence region near the N-terminus, but beyond the first 30 residues (Köhler et al 1993). It might also be present in the LMW-m type, although it was not present according to the DNA sequences for this type of subunit. The DNA sequences should not be considered definitive at this time. All clones reported so far (see Cassidy and Dvorak 1991; D'Ovidio 1992a,b) indicate a cysteine at position 5, whereas the actual sequencing results of Lew et al (1992) demonstrate that there are variations of the LMW-m type sequence in which either arginine or histidine is substituted for the cysteine at position 5. Furthermore, no DNA clone has yielded a sequence that begins with Ser-His-Ile-, which is in fact the beginning sequence of the major LMW-GS type defined in this work and earlier studies (Tao and Kasarda 1989, Lew et al 1992). The presence of the C^{b*} cysteine and the C^x cysteine (Köhler et al 1993) would make the LMW-s subunit a chain extender despite the absence of the cysteine at position 5. We point out that the disulfide linkages defined by Köhler et al (1993) cannot be defined as belonging to LMW-s or LMW-m types, the enzymatic cleavages necessary for obtaining disulfide-linked peptides detached these peptides from their defining N-terminal sequences so that the C^{b*} cysteine might well appear in either or both types.

In addition to the LMW-s and LMW-m types of subunits, we found significant amounts of subunits with N-terminal sequences similar to those of α-type and γ-type gliadins. We have encountered some reluctance among cereal chemists to accept these gliadin-like components found in glutenin as true glutenin subunits (various personal communications). If the definition of glutenin components is that they are proteins linked by intermolecular disulfide bonds, and the definition of gliadins is that they are monomeric proteins consisting of a single polypeptide chain (and we contend that this is the most workable definition of gliadins and glutenins), then these gliadin-like components of glutenin should be considered glutenin subunits even though they are almost certainly coded at the *Gli*-1 and *Gli*-2 loci rather than the *Glu*-3 loci.

In support of this contention, we point out that Köhler et al (1993) have defined a disulfide-linked pair of peptides from a glutenin digest in which one peptide of the pair is derived from a γ gliadin and the other from a LMW-GS (as defined by the DNA sequences corresponding to LMW-m type subunits). The cysteine of the γ-type sequence had not been defined by DNA sequences of γ gliadins, but was presumably an extra cysteine that had resulted from mutation of a codon for phenylalanine into one for cysteine. Furthermore, α- and γ-type sequences found

in highly purified (by ion-exchange in the presence of 6*M* urea) glutenin preparations by Lew et al (1992) had marker sequence differences from those of normal gliadins that defiined them as gliadin mutants with, in each case, a single extra cysteine. This would make them glutenin subunits and, furthermore, likely chain terminators of growing glutenin polymers. Masci et al (1993) have shown also that D glutenin subunits are in fact mutated ω-gliadins that have acquired at least one cysteine residue.

If we consider the two main groups of sequences found, namely the sequences peculiar to LMW-GS (LMW-s and LMW-m) and the gliadin type sequences (α-type and γ-type), it is noteworthy that these latter make up 43% in Lira 42, but only 20% in Lira 45 (see Table II). The large amount of these likely chain terminators in the Lira 42 (type 1) glutenin is likely to be a factor in determining the poorer quality of the type 1 line, contributing to a lower size range of glutenin polymers in Lira 42. Since α-type gliadins are most probably coded at the *Gli*-2 loci, not linked to the *Gli*-1/*Glu*-3 loci, the particular situation described here could not be common to the other LMW-GS type 1 and type 2 durum wheats and could reflect a different degree of expression of these α-type genes between the two biotypes. However, in the case of cultivar Lira, the presence of a rather high amount of α- and γ-type sequences would enhance the effect of lesser amounts of the key chain-extending subunits, LMW-s and LMW-m, in decreasing the molecular weight distribution of the glutenin polymers in Lira 42.

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