D-Glutenin Subunits: N-Terminal Sequences and Evidence for the Presence of Cysteine[1]

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Cereal Chem. 70(5):581–585

ABSTRACT

The two-chromosome, 1D-coded D subunits of low molecular weight glutenins from Chinese Spring wheat were purified by using preparative isoelectric focusing and reversed-phase high-performance liquid chromatography. Their N-terminal sequences corresponded to those of the two-chromosome, 1D-coded ω-gliadins, which they also resemble in molecular weight and isoelectric point. In order to show the presence of cysteine in D subunits, which should account for their presence in glutenin, the reduced subunits were reacted with a fluorogenic reagent specific for sulfhydryl groups in a mixture with the 1D-controlled ω-gliadins that were assumed to have no cysteine residues. When the mixture was separated in a reversed-phase high-performance liquid chromatography system equipped with UV absorbance and fluorescence detectors in series, all components were detected by UV absorbance, but only the peaks corresponding to the D subunits showed fluorescence. This confirmed the presence of at least one cysteine in the D glutenin subunits. If only one cysteine residue is present, which seems likely, it is possible that D subunits would negatively influence quality characteristics because they would be able to form only intermolecular disulfide bonds, thereby acting as terminators of the growing glutenin polymers.

Wheat storage proteins are made up of gliadins and glutenins. Both these classes of proteins are extensively studied because of their importance in determining wheat quality characteristics. Gliadins are monomeric proteins; disulfide bonds, when present, are only intramolecular. They are coded by loci on the short arms of groups 1 and 6 chromosomes (*Gli-1* and *Gli-2*, respectively). In contrast, glutenins are polymers formed from protein monomers (subunits) linked by intermolecular disulfide bonds. On the basis of their mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the monomers are usually classified into two groups: high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) (Bietz et al 1975). HMW-GS are made up of proteins coded by genes on the long arms of group 1 chromosomes (*Glu-1* loci). They have also been called A subunits (Payne and Corfield 1979). LMW-GS are sometimes subdivided further into B and C subunits on the basis of their SDS-PAGE mobilities. The B group is slower moving with apparent molecular weights of 40,000–50,000. The faster moving C subunits have apparent molecular weights of about 30,000–40,000. Thus, the A, B, and C designations are based on their relative mobilities in SDS-PAGE (slower to faster, respectively).

An additional group of LMW-GS, migrating slightly more slowly than the B group, are called the D glutenin subunits, presumably because they were discovered after assignment of the A, B, and C designations (Payne and Corfield 1979). The B and C subunits are coded mainly by genes of the *Glu-3* loci, but also by some at the *Gli-1* loci. Both loci are on the short arms of group 1 chromosomes (Lew et al 1992). At least three D subunits are detectable in Chinese Spring, one of which is coded by a gene on the short arm of chromosome 1B. The other two are coded by genes on the short arm of chromosome 1D (Jackson et al 1983). The proteins coded by the latter two genes have the molecular weight and isoelectric pH characteristics of 1D-coded ω-gliadins (Masci et al 1991a,b). It is possible that the 1D ω-gliadins and 1D-coded D glutenin subunits originate from genes at the same locus because no recombination has been found (Payne et al 1986).

In a previous study, Masci et al (1991a) hypothesized that D subunits could be mutated ω-gliadins because they have similar electrophoretic characteristics, both in isoelectric focusing (IEF) × SDS-PAGE and in acid-polyacrylamide gel electrophoresis (A-PAGE) × SDS-PAGE. In a subsequent work, Masci et al (1991b) found that D subunits are present only in those bread wheat cultivars with 1D-coded ω-gliadins similar to Chinese Spring (Chinese Spring-type wheats). They are absent from all the cultivars with ω-gliadins similar to Cheyenne (Cheyenne-type wheats).

Little is known about these proteins. The aim of this work was to better characterize them, both by determining their N-terminal amino acid sequences and by showing the possible presence of cysteine in their primary structures.

MATERIAL AND METHODS

IEF × SDS-PAGE

Extraction of proteins from Chinese Spring flour and two-dimensional analysis were performed according to Kasarda et al (1988), with the exception that, in the gel solution, we used 2% pH range 3.5–10, 1% pH range 2.5–4, and 2% pH range 4–6 ampholytes (Pharmacia-LKB, Uppsala, Sweden). A Mini-Protean II 2-D Cell (Bio-Rad, Richmond, CA) was operated according to the instruction manual.

Preparation of Wheat Storage Proteins for Free-Flow IEF

Flour (1 g) from the cultivar Chinese Spring was extracted with 5 ml of 50% *n*-propanol containing 50 m*M* dithioerythritol for 1 hr at 60°C with gentle magnetic stirring. After centrifugation at 10,000 × *g* for 10 min, the supernatant was made 60% propanol and left at 4°C overnight. In these conditions, HMW-GS precipitate along with HMW albumins and 1B-ω-gliadins after centrifugation (Marchylo et al 1989). This step was necessary because HMW-GS coelute with D subunits in reversed-phase high-performance liquid chromatography (RP-HPLC).

The supernatant, containing 1D-coded ω-gliadins and D subunits, along with α-, β-, and γ-gliadins and LMW-GS (about 70 mg of protein), was mixed with 1% ampholytes (Serva, Westbury, NY) pH range 3–5 and 1% ampholytes (Bio-Rad) pH range 5–7 and made up to 55 ml with 60% *n*-propanol. It was loaded in a Rotofor (Bio-Rad) apparatus without urea or detergent (Curioni et al 1990). The anode and cathode solutions were 0.1*M* sulfuric acid and 0.1*M* ethanolamine, respectively. The preparative IEF was run at 12 W of constant power for 4 hr at 8°C. Twenty fractions were collected. After the relative pH was measured, the fractions were analyzed by SDS-PAGE using the acrylamide solutions and conditions described by Werner et al (1992), except

[1]This work was partly supported by the ECLAIR Programme of the European Communities, Contract AGRE 0052.

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The mention of firm names or trade products does not imply that they are endorsed or recommended by the USDA over other firms or similar products not mentioned.

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that glycerol was omitted. Gels were stained with Coomassie Brilliant Blue G-250 according to Neuhoff et al (1988) without the fixation step.

RP-HPLC Purification of D Subunits

Fractions of interest were further purified by RP-HPLC. We used a Spectra-Physics SP8700 solvent delivery system (Spectra-Physics, San Jose, CA) connected to an Isco V4 detector (Isco, Lincoln, NE) and a SP4270 integrator (Spectra-Physics). Separation of proteins was performed on a Vydac C_{18} semipreparative, 10-mm × 25-cm column (Vydac, Hesperia, CA) equilibrated at 50° C for 40 min with a linear gradient of 30–38% aqueous acetonitrile containing 0.05% trifluoroacetic acid at a flow rate of 1.5 ml/min. Initial conditions were held for 10 min before starting the gradient. After detection at 210 nm, peaks corresponding to D subunits, as indicated by electrophoretic analysis, were collected and concentrated to about 0.1 ml on a Savant Speed Vac concentrator (Savant, Farmingdale, NY) for alkylation with the fluorescent probe.

Detection of the Fluorescent Adducts

The chromatographic system just described was modified by the addition of a spectrofluorescence detector with a xenon source (model FL-750BX, McPherson Corp., Acton, MA) connected in series with the UV absorption detector and monitored by a strip chart recorder (model 165, Linear Corp., Reno, NV).

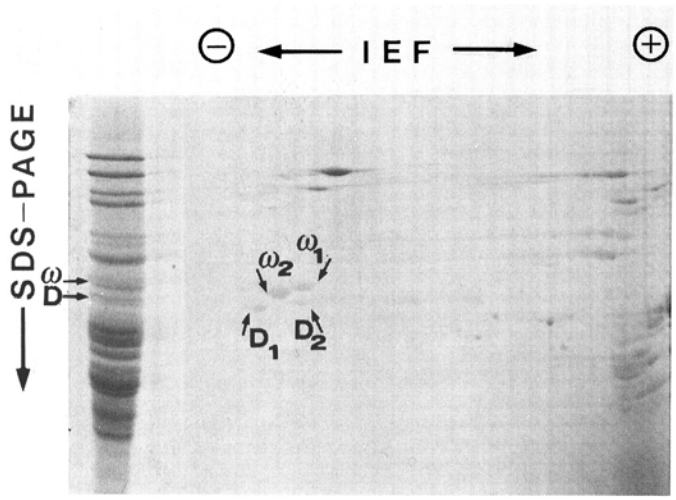

Fig. 1. Isoelectric focusing (IEF) × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Chinese Spring. One-dimensional reference (left) shows resolution of 1D-coded ω-gliadins and D glutenin subunits using this faster technique. The pH range is from 4.5 (acid side) to 7 (basic side).

We used the fluorogenic alkylating agent 7-fluoro-4-sulfamoyl-2,1,3-benzoxadiazole (ABD-F, Wako Chemicals, Richmond, VA) to alkylate sulfhydryl groups. This reagent is specific for thiols and specifically labels the cysteine side chains of proteins. The reaction is stoichiometric and is suitable for quantification of sulfhydryl groups (Kirley 1989). In addition to its specificity, we chose ABD-F for its fast reaction rate and strong fluorescence under our RP-HPLC separation conditions. The ABD-F conjugate with sulfhydryl groups has a maximum fluorescence near pH 2 (Toyo'oka and Imai 1984), which is the pH of the solvent system we employed.

Reduction and alkylation of disulfide bonds were performed in a single step according to a modification of the technique proposed by Kelso et al (1991). Protein samples for reaction with ABD-F were obtained by RP-HPLC fractionation. They corresponded to: single D subunits (purified by preparative IEF); a mixture of the two D subunits; or a mixture of ω-gliadins and purified D subunits. Protein amounts in the samples obtained from RP-HPLC and concentrated in the Speed Vac ranged from 30 pmol to 1 nmol in 100 μl of solvent. The samples were diluted with 500 μl of 0.1*M* borate buffer made of 0.8 m*M* ethylenediaminetetraacetic acid (pH 8.8) containing an excess of ABD-F (2–6 nmol dissolved in water before addition to buffer) and 1 μl of 99% tributylphosphine (Aldrich Chemical, Milwaukee, WI) as a reducing agent. The reaction was performed at 50° C for 10 min. After the reaction mixture had cooled to room temperature and had been filtered through a 0.45-μm filter, each sample was injected into the HPLC system, which was equipped with both absorbance and fluorescence detectors, and eluted with the same gradient used for purification. The fluorescent adducts were detected by excitation at a wavelength of 385 nm; emission was monitored at 515 nm. Peaks corresponding to D subunits were recollected and concentrated for N-terminal sequence analysis and characterization by SDS-PAGE.

N-Terminal Sequences of D Subunits

To determine the N-terminal amino acid sequences, we used a pulsed-liquid phase amino acid sequencer (model 477A, Applied Biosystems, Foster City, CA) with an on-line phenylthiohydantoin-amino acid analyzer (model 120A). Fractions containing the proteins of interest were applied to a glass-fiber filter pretreated with 3.0 mg of Biobrene Plus (Applied Biosystems). Analyses of the data were performed both by the instrument data analysis program and by the operator.

RESULTS

The total two-dimensional pattern of wheat storage proteins in Chinese Spring is shown in Figure 1. The 1D-coded ω-gliadins and the D glutenin subunits have very acidic pIs. We used an ampholyte combination that produced a pH range of 4–8 so that

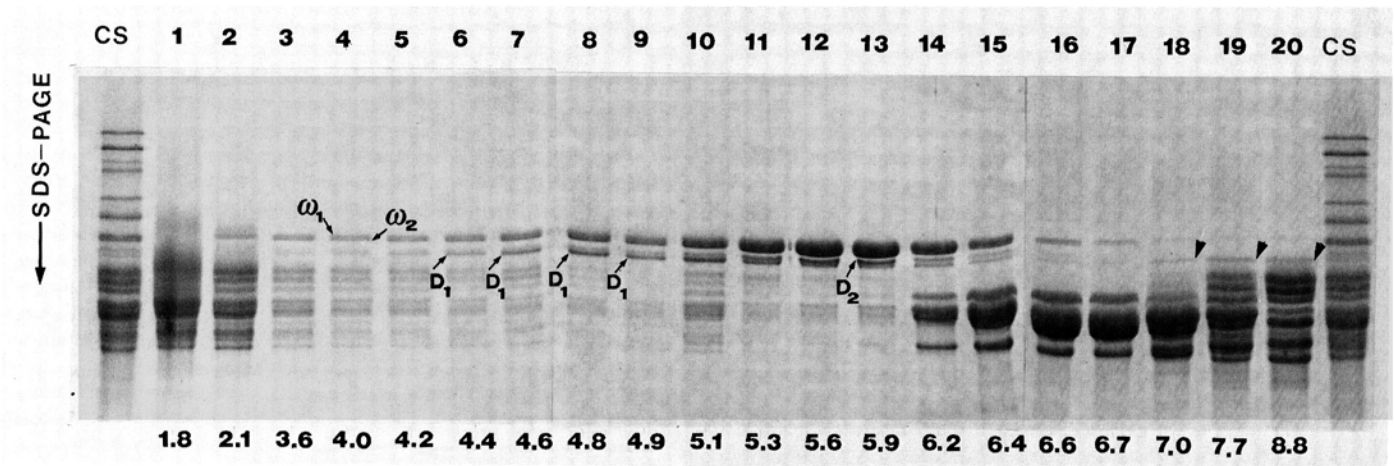

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Rotofor fractions showing resolution of 1D-coded ω-gliadins and D glutenin subunits. Single fraction pH values indicated at bottom. CS = Chinese Spring.

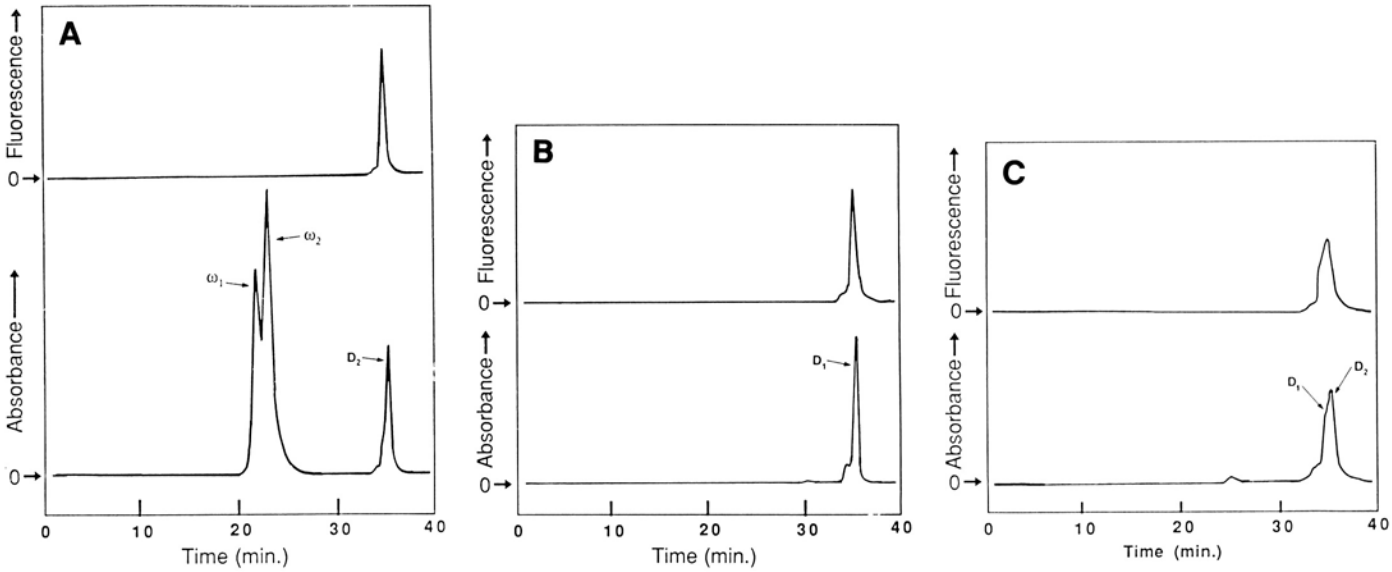

Fig. 3. Chromatograms obtained after UV absorbance (lower) and fluorescence emission intensity (upper). **A,** D_1 glutenin subunits and ω-1 and ω-2 gliadins loaded together. **B,** D_2 glutenin subunit. **C,** D_1 and D_2 glutenin subunits loaded together.

only the acidic proteins were well focused. All the other proteins migrated to the basic side of the gel.

As a first step, we analyzed, by RP-HPLC, a total protein extract from Chinese Spring that had first been freed of HMW-GS, HMW albumins, and 1B-coded ω-gliadins. SDS-PAGE of the proteins in the resulting peaks indicated that the two 1D-controlled D subunits largely eluted together with slight separation, as indicated by a shoulder on the peak (Fig. 3c). The proteins in this peak were collected and alkylated with vinylpyridine, according to the procedure of Lew et al (1992), to detect any possible cysteine residues; they were then subjected to amino acid sequencing through 10 cycles of the Edman degradation. The result indicated a mixture of two specific components with N-terminal sequences, similar to those of ω-1 and ω-2 gliadins from Chinese Spring (Kasarda et al 1983), except that the third cycle showed leucine and glutamine instead of leucine and glutamic acid, which indicated a substitution of glutamine for glutamic acid in the third position of the sequence corresponding to ω-2 gliadin.

Because the two D subunits have different pIs, we exploited this characteristic for further purification. After free-flow IEF, the 20 fractions were checked on SDS-PAGE (Fig. 2). The relative pHs were also measured, and they are indicated in Figure 2. Fractions 6 and 7, corresponding respectively to pH 4.40 and 4.6, were enriched in the fastest D subunit, which we refer to as D_1; consequently, they were used for further purification. Fraction 13 (pH 5.9) was used for purification of the D_2 glutenin subunit, the slowest one. It is interesting to note that fractions 18, 19, and 20 show a band with the same SDS-PAGE mobility as that of the D_1 component; however, it had a completely different pI (arrows in Fig. 2). This band probably corresponds to a component that has not yet been characterized.

The IEF separation was effective in separating the D subunits from one another but not from all other proteins. Accordingly, the final purification of the two subunits was accomplished by RP-HPLC, which separated the D subunits from other proteins.

The purified D_2 component and a mixture of the D_1 component with ω-1 and ω-2 gliadins were treated with ABD-F as described above.

We used the 1D-coded ω-gliadins as a control in demonstrating differences between these cysteine-free proteins and the D glutenin subunits. In Figure 3a, the fluorescence of the ABD-F treated D_1 glutenin subunit and the absence of fluorescent ω-1 and ω-2 gliadin components (not separated from one another), can be seen in chromatograms obtained by simultaneous detection of UV absorption and fluorescence emission. In Figures 3b and 3c, the chromatograms show that both the D_1 and D_2 glutenin subunits were fluorescent after treatment with ABD-F. These results demonstrate the presence of cysteine in the D glutenin subunits and its absence from the ω-1 and ω-2 gliadin components.

omega-1	K E L Q S P Q Q S F
D_1	K E L Q S P Q Q S F
omega-2	A R E L N P S N K E L Q S P Q Q S F
D_2	A R Q L N P S N K E

Fig. 4. Comparison of N-terminal amino acid sequences for 1D-coded D glutenin subunits and 1D-coded ω-gliadins (Kasarda et al 1983). Differences are underlined. Single-letter code for amino acids: A, alanine; E, glutamic acid; F, phenylalanine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine.

In this initial study, we did not attempt any rigorous quantitative analysis of the amounts of cysteine in the D glutenin subunits. However, analysis of the mixture of the two D glutenin subunits (Fig. 3c) showed no change in the relative intensities of the main peak (corresponding to the D_1 component) and the shoulder (corresponding to D_2 component) when chromatograms obtained simultaneously by the two detection methods were compared. This result makes it likely that the two subunits have the same number of cysteine residues. If there were a difference, it seems likely that the two subunits would fluoresce with intensities proportional to the number of cysteines.

We used peaks D_1 and D_2, collected after the simultaneous analysis of UV absorbance and fluorescence emission intensity, for the N-terminal sequence (Fig. 4) and SDS-PAGE analyses (Fig. 5). We found that the first 10 amino acid residues from the N-terminus of the D_1 component coincided exactly with those of the Chinese Spring ω-1 gliadin. The equivalent sequence from the D_2 component coincided with that of the Chinese Spring ω-2 gliadin, with the exception of position 3, where glutamine, rather than glutamic acid, was found. This single difference could be a consequence of a point mutation in the triplet coding for glutamic acid (or glutamine). It could also be the result of deamidation of glutamine in the ω-2 gliadin during the preparation procedure, but this does not seem likely. The glutamine is present in the third position of ω-gliadins from *Triticum durum* and *T.*

monococcum that have N-terminal sequences that are closely similar to those of the ω-2 gliadin of Chinese Spring (Kasarda et al 1983).

DISCUSSION

It seems likely that differences in mixing and baking quality among varieties would result from differences in the native glutenin fraction. The number and positions of cysteine residues in glutenin subunits must play an important role in determining the properties of native glutenin (Lew et al 1992) and, thereby, in determining mixing and baking quality. Consequently, developing methods for identifying and quantifying these cysteine residues is of considerable importance. The reagent of Toyo'oka and Imai (1984), which we have adapted in this work for analysis of cysteine in glutenin subunits, appears to be a useful tool for detecting even small amounts of cysteine in glutenin proteins or in peptides derived from these proteins by enzymatic or chemical degradation (Kirley 1989). Although we have not carried out rigorous quantitative studies, the smallest amount analyzed was 30 pmol of a D glutenin subunit. About one-third less protein could have been detected. The D glutenin subunits may have only one cysteine residue in each protein molecule and, if so, proteins with more than one cysteine residue would show greater sensitivity.

In regard to the potential role of D glutenin subunits in determining quality characteristics, it may be noted that quality evaluation (by the SDS sedimentation test) of the two biotypes present in the cultivar Newton (differing only in the presence or absence of D subunits in the 1D-coded ω-gliadins and one LMW-GS) indicated better quality for the biotype with no D subunit (Masci et al 1991a). Also, as noted by Sozinov and Poperelya (1982), the *Gli-D1* block that apparently corresponds to the Cheyenne-type ω-gliadins has better quality associated with it than the block that corresponds to Chinese Spring-type ω-gliadins. Masci et al (1991b) found that most wheat cultivars have one or the other type of ω-gliadin pattern; the Cheyenne-type does not have D subunits, whereas the Chinese Spring-type does. These observations provide some indirect evidence that the presence of D glutenin subunits in a variety makes a negative contribution to wheat mixing and baking quality. We note, however, that this may be the case only for varieties without excessive strength and elasticity.

It is generally accepted that ω-gliadins do not have any sulfur-containing amino acids. This characteristic has been used to separate ω-gliadins from α-, β-, and γ-gliadins, which do have cysteine residues, by passing the mixture (after reduction of the proteins) through a column matrix with free sulfhydryl groups (Egorov et al 1986, Odintsova et al 1986). The α-, β-, and γ-gliadins bound to the column by forming disulfide linkages with the matrix; the ω-gliadins passed through the column and were recovered in the eluate. Our finding of ω-type N-terminal amino acid sequences for D subunits, along with cysteine residues in these subunits, changes the picture. It appears likely that the D subunits formed as a consequence of a mutation of an ω-gliadin gene (or genes), such that one or more cysteine codons was produced. Of course, it cannot be ruled out that the reverse change occurred; that is, a D glutenin subunit was the ancestral form that gave rise to genes coding for cysteine-free ω-gliadins through loss or change of a cysteine codon. However, the ω-gliadins are plentiful in wheat and D subunits are scarce, so it seems somewhat more likely that genes for the former gave rise to genes for the latter.

How can the potential negative effects of D glutenin subunits be explained? We offer the following speculations.

Because we have not yet quantified the number of cysteine residues in D subunits, we have no proof that there is only one cysteine per subunit, but we suggest that this is a likely possibility—more likely than an evolutionary jump forming two or more cysteine codons. Additionally, the presence of a single cysteine could explain the negative effect of D subunits on dough quality. If each D subunit could form only one intermolecular disulfide bond, D subunits would tend to decrease the size of glutenin polymers relative to a subunit that could form two or more intermolecular disulfide bonds, which is a requirement for extending glutenin polymers during their synthesis. Thus, the D subunits might play a role in determining quality characteristics that are influenced by the average size of the glutenin polymers. This proposed effect of D glutenin subunits to decrease the average molecular weight of glutenin polymers is similar to that proposed (Kasarda 1989, Tao and Kasarda 1989, Lew et al 1992) for certain α- and γ-type glutenin subunits that have apparently resulted from mutation of a serine codon to a cysteine codon in ancestral α- and γ-type gliadin genes.

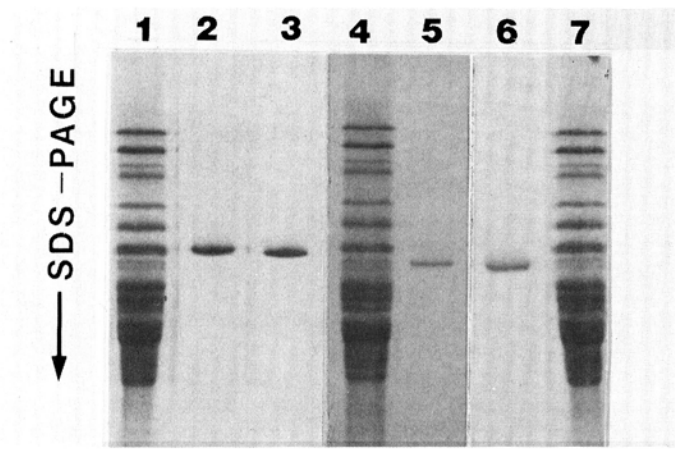

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total protein extract from Chinese Spring; ω-1 gliadin; ω-2 gliadin; total protein extract from Chinese Spring; D_2 glutenin subunit; D_1 glutenin subunit; and total protein extract from Chinese Spring (lanes 1–7, respectively). Gels run using purified proteins collected after simultaneous analysis of UV absorbance and fluorescence emission intensity.

ACKNOWLEDGMENTS

We dedicate this article to Salvatore Auricchio of Naples, Italy, on the occasion of his 60th birthday.

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[Received February 1, 1993. Accepted May 24, 1993.]