Isolation and Characterization of Gliadin-Like Subunits from Glutenin

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ABSTRACT

The polypeptide subunits of wheat glutenin obtained after reductive cleavage of disulfide bonds have been separated into two fractions by a modified Osborne technique. Approximately 62% of the subunits by weight are soluble, as is gliadin, in neutral 70% ethanol; the ethanol-soluble subunits are mainly of 44,000 molecular weight (MW). The ethanol-insoluble glutenin subunit fraction is markedly different in amino acid composition, and consists of high-MW subunits, and some of lower MW. Sodium dodecyl sulfate electrophoresis, starch-gel electrophoresis, and amino acid analyses suggested that some ethanol-soluble subunits of reduced glutenin may be equivalent to subunits of high-MW gliadin. To test this possibility, both fractions were reduced and alkylated and 44,000-MW subunits were isolated and partially characterized.

Wheat gluten is formed by the interaction of endosperm proteins with other cellular components when flour is hydrated and becomes the elastic cohesive mass known as dough. The major proteins responsible for the properties of gluten may be fractionated by Osborne's (1) procedure into gliadin, which is soluble in neutral 70% ethanol, and glutenin, the alcohol-insoluble component. Gliadin consists mainly of many different single-chained proteins of relatively low molecular weight (MW), whose conformations are stabilized by intramolecular disulfide bonds (see 2, 3, and 4 for recent studies and reviews of earlier work). A small amount of high-MW protein has also been separated from gliadin by gel-filtration chromatography (4). Glutenin, however, contains subunits of many sizes joined through disulfide bonds into proteins with MWs ranging into the millions (see 5 for review). Glutenin's subunits have been partially separated and characterized after reduction of disulfide bonds (6–10). We also recently studied the number and MW of gluten proteins and their subunits by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (11).

Similarities between glutenin's subunits and gliadin molecules (see 12–13 for review) have led to theories that glutenin consisted, at least in part, of gliadin-like subunits joined by intermolecular disulfide bonds; other studies, however, emphasized differences between gliadin and glutenin. This apparent discrepancy was partially resolved by SDS-PAGE (11), which showed that many glutenin subunits are unique, but at least three correspond in MW to proteins of gliadin. Prolamin-like subunits have also been observed in glutelins of corn (14–18), barley (19), and sorghum (20); for these cereals, fractionation of glutelin subunits was achieved by alcohol extraction in the presence of mercaptoethanol.

Since, to our knowledge, no attempt had been made to fractionate wheat glutenin subunits by differential solubility in solvents of different polarity, we undertook the present study. This investigation demonstrated that certain glutenin subunits are soluble in neutral dilute ethanol; furthermore, these subunits are very similar to the subunits of high-MW gliadin.

[1]Agricultural Research Service, U.S. Department of Agriculture. Mention of firm names or trade products does not constitute endorsement by the Department over others of a similar nature not mentioned.

MATERIALS AND METHODS

Protein Samples

Glutenin and gliadin were isolated from defatted Ponca hard red winter wheat flour as described previously (11). SDS-PAGE revealed none of the high-MW subunits characteristic of reduced glutenin in reduced gliadin. By a similar method (21), SDS-PAGE revealed no proteins with mobilities characteristic of gliadin in unreduced glutenin.

High-MW gliadin (fractions a and b in Fig. 3, ref. 11) was isolated from whole gliadin by gel filtration on a 4.2 × 93-cm. column of Sephadex G-100 (Pharmacia, Inc., Uppsala, Sweden). High-MW gliadin fractions eluting from the column are characteristically turbid, possibly because of protein association, Sephadex breakdown products (22), or because of carbohydrates or lipids extracted with the proteins. Some high-MW gliadin preparations contained as little as 5 to 6% nitrogen. Centrifugation of high-MW gliadin suspended in 1.0% acetic acid for 60 min. at

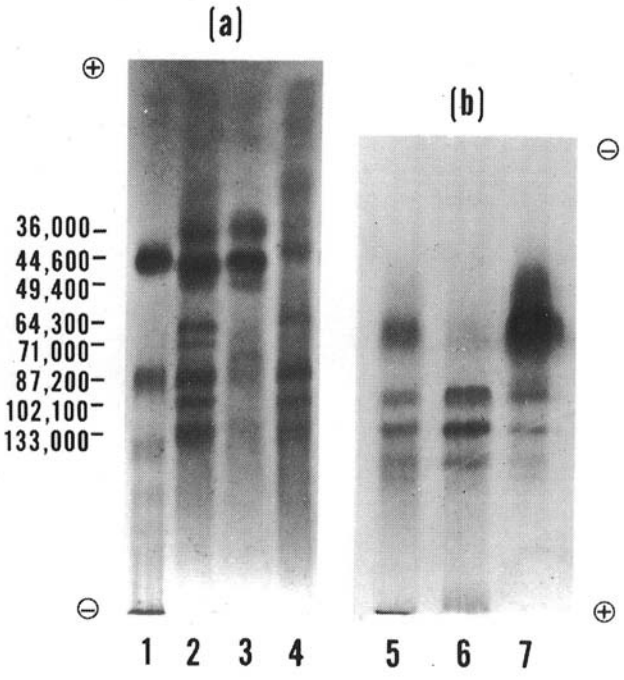

Fig. 1. (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of 1, molecular weight (MW) calibration mixture of monomeric and polymeric ovalbumin; 2, whole reduced glutenin; 3, ethanol-soluble *S*-aminoethyl (AE)-glutenin; and 4, ethanol-insoluble AE-glutenin. MWs of major glutenin subunits, determined previously (11), are indicated. (b) Starch-gel electrophoretic patterns of 5, AE-glutenin; 6, ethanol-insoluble AE-glutenin; and 7, ethanol-soluble AE-glutenin.

54,000 × g produced a small brownish pellet, a clear solution, and a fragile, milky surface layer. The clear solution contained essentially all the protein (over 14% nitrogen on a solids basis). High-MW gliadin clarified in this manner was used for all studies.

S-Aminoethyl (AE), *S*-pyridylethyl (PE), and *S*-cyanoethyl (CN) derivatives of reduced proteins were produced as described by Rothfus and Crow (7), Friedman et al. (23), and Crow and Rothfus (6).

Electrophoretic and Chromatographic Methods

SDS-PAGE was performed in horizontal slabs by the method of Koenig et al. (24); experimental details were described previously (11). Starch-gel electrophoresis with 3M urea-aluminum lactate buffer at pH 3.1 was conducted as described by Huebner et al. (25).

Ethanol-soluble AE-glutenin subunits were complexed with SDS by incubation in 1.0% acetic acid for 5 min. at 95°C. with twice their weight of SDS; for high-MW gliadin, 1.0% 2-mercaptoethanol was also included during complexing to reduce disulfide bonds. The subunits (20 to 30 mg. protein per ml.) were then fractionated by upward flow gel filtration on Sephadex G-200 (5.0 × 87.4 cm., particle size 40 to 120μ). The column was eluted with 0.1% SDS-1.0% acetic acid at 40 ml. per hr.; 5.0-ml. fractions were collected, and absorbance was monitored at 280 nm. SDS was removed from proteins after chromatography as described by Weber and Kuter (26), except that 2-mercaptoethanol was omitted for AE-glutenin subunits. Quantitative removal of SDS was demonstrated by cathodic migration of the subunits at pH 3.1 upon starch-gel electrophoresis. Recoveries were 70 to 80%.

Proteins were hydrolyzed for 24 hr. in 6N HCl at 110°C. in tubes sealed *in vacuo* prior to amino acid analysis on a Beckman analyzer by a two-column accelerated system (27). Amino acid compositions were calculated automatically (28); no corrections were made for destruction or slow liberation of amino acids during hydrolysis. AE-cysteine, when present, eluted with lysine or histidine, depending on column length. Deionized water and analytical grade reagents and chemicals were used for all studies.

RESULTS

Fractionation of Reduced and Alkylated Glutenin

Attempts were first made to fractionate AE-glutenin subunits by differential solubility in 70% ethanol, in a manner analogous to that in which gliadin is separated from glutenin (1). In a typical experiment, 1.025 g. AE-glutenin was stirred with 300 ml. 70% ethanol-0.7% acetic acid; 3.8% of the material was removed by centrifugation at room temperature. The clear solution was adjusted to pH 6.60 with 2N NaOH and stored in the refrigerator overnight. A copious white precipitate was separated by centrifugation at 4°C.; the pellet was washed, and supernatants were combined. Ethanol was removed from the soluble fraction under reduced pressure in a rotary evaporator, and both fractions were dialyzed vs. dilute acetic acid and lyophilized. Total recovery was 87.8%; the remained may be accounted for by losses during transfer and dialysis, and by complete drying of the recovered proteins upon lyophilization. Of the recovered material, 38% by weight precipitated from 70% ethanol at pH 6.6, the remainder (62%) being soluble under these conditions. Similar results were obtained in duplicate experiments, and also for CN- and PE-glutenin.

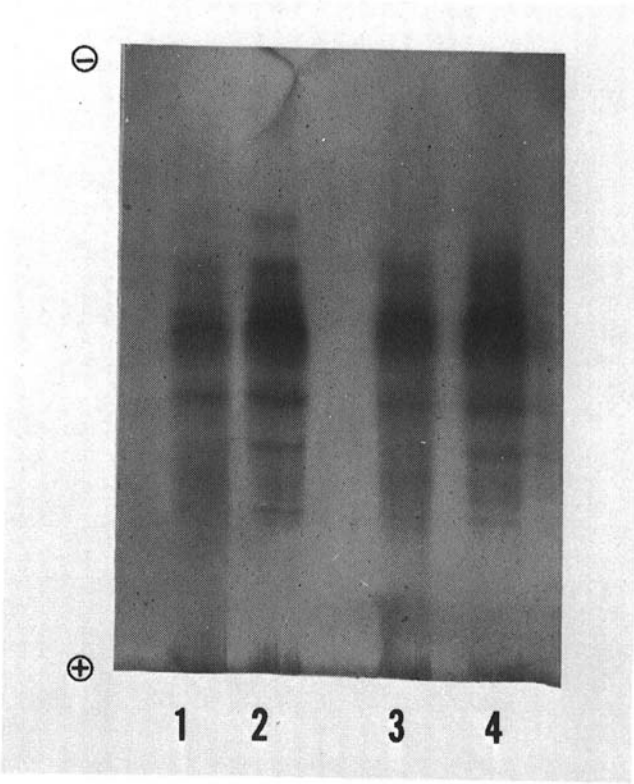

Fig. 2. Starch-gel electrophoretic patterns of 1, *S*-pyridylethyl (PE)-high-MW gliadin; 2, ethanol-soluble PE-glutenin; 3, AE-high-MW gliadin; and 4, ethanol-soluble AE-glutenin.

The fractions obtained by this procedure, which will be referred to as ethanol-soluble and -insoluble subunits, were examined by SDS-PAGE and starch-gel electrophoresis (Fig. 1). The ethanol-soluble fraction contained mainly 44,600 and 36,000 MW subunits (MWs were determined previously) (11); these primarily correspond to subunits of greatest mobility in the starch gel. Small amounts of 49,400 MW subunits, as well as several minor ones, also occur in the ethanol-soluble fraction. The ethanol-insoluble fraction differs markedly from the soluble one and is particularly enriched in the highest MW subunits (MW 84,200 to 133,000), which migrate most slowly upon starch-gel electrophoresis. The insoluble fraction also contains several low- and intermediate-MW subunits, but lacks polypeptides with mobilities corresponding to the major components of the soluble fraction.

The 36,000 and 44,600 MW subunits of ethanol-soluble glutenin have SDS-PAGE mobilities identical to those of polypeptides in whole gliadin; in particular, ethanol-soluble glutenin resembles high-MW gliadin in number, distribution, and MW of subunits (see Fig. 3 in ref. 11). (Since the calculated MW differences between the corresponding subunits of gliadin and glutenin are within the range of

variation of the method, they will now be referred to as 44,000 and 36,000 MW subunits.)

To further examine these fractions, the AE and PE derivatives of high-MW gliadin were compared to ethanol-soluble AE- and PE-glutenin by starch-gel electrophoresis (Fig. 2). Several subunits with identical mobilities occurred in both fractions. Some ethanol-soluble glutenin subunits, however, were absent in high-MW gliadin.

Amino acid analyses of ethanol-soluble and -insoluble AE-glutenin are presented in Table I, along with compositions of glutenin, gliadin, and high-MW gliadin. Ethanol-soluble AE-glutenin differs significantly in composition from the ethanol-insoluble fraction, especially in the contents of glycine, aspartic acid, alanine, tyrosine, and phenylalanine. Soluble AE-glutenin and high-MW gliadin, whose subunits have similar MWs and charge distributions, also have very similar amino acid compositions.

TABLE I. AMINO ACID ANALYSES OF ETHANOL-SOLUBLE AND -INSOLUBLE AE-GLUTENIN, AND COMPARISON TO OTHER GLUTEN PROTEINS[a]

Amino Acid	Insoluble AE-Glutenin	Soluble AE-Glutenin	High-MW Gliadin	Purified Gliadin	Glutenin[b]
Lysine	2.8[c]	2.7[c]	1.0	0.6	1.4
Histidine	1.3	1.3	1.4	1.9	1.5
Arginine	2.7	2.2	2.6	1.9	2.2
Aspartic acid	3.2	1.4	2.0	2.5	2.7
Threonine	3.2	2.4	2.6	1.8	3.1
Serine	6.6	7.2	7.0	5.1	5.9
Glutamic acid	32.7	39.4	37.5	39.4	32.8
Proline	11.0	15.0	15.1	16.3	13.5
Glycine	12.9	3.3	4.9	2.5	9.2
Alanine	4.1	2.4	2.7	2.9	4.0
Half-cystine	0.0	0.0	1.3	2.1	1.4
Valine	3.5	4.3	4.0	4.2	4.8
Methionine	0.8	1.3	1.3	1.1	1.4
Isoleucine	2.3	3.7	3.4	3.9	3.3
Leucine	6.1	7.5	7.4	7.4	6.7
Tyrosine	4.2	1.5	1.7	2.0	2.7
Phenylalanine	2.5	4.5	4.1	4.4	3.2

[a]Compositions are expressed as mol. %. AE = S-aminoethyl; MW = molecular weight.

[b]Adapted from Wu and Dimler (29).

[c]Includes AE-cysteine.

Other Fractionation Experiments

To optimize conditions for fractionation of glutenin subunits, and since aminoethylation may modify their solubility properties, the pH of a solution of AE-glutenin in 70% ethanol-0.7% acetic acid was increased in small steps by addition of 2N NaOH. The precipitate formed at each pH increment was removed by centrifugation. Most precipitation occurred between pH 5.5 and 5.8; only slight amounts precipitated between pH 4.6 and 5.5, and between 5.8 and 7.4, above which no further precipitation occurred. SDS-PAGE showed all precipitates to be similar in subunit composition. For this reason, and since at pH 6.6 precipitation was more than 99% complete, this pH is a logical choice for the fractionation. It is likely that no further fractionation can be achieved by varying the pH.

The effect of ionic strength (μ) on the ethanol fractionation of glutenin subunits was also examined. Fractional salt precipitation has been applied to AE-glutenin

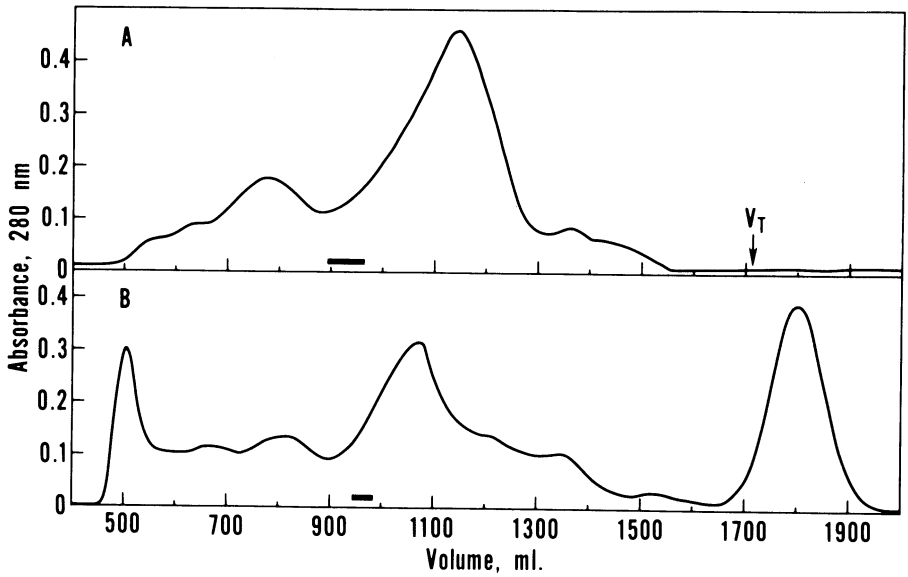

Fig. 3. Sephadex G-200 elution patterns of A, 312 mg. of ethanol-soluble AE-glutenin; and B, 235 mg. of reduced high-MW gliadin. Tubes which were combined into 44,000 MW fractions are indicated on the abscissa. Total column volume (V_T) is indicated. The peak in B eluting after V_T contained only nonproteinaceous material.

(7), and there is some similarity between ethanol-insoluble AE-glutenin and salt fraction A and between ethanol-soluble AE-glutenin and salt fraction B (see Fig. 6 in ref. 11). The solvent fractionation in 70% ethanol uses μ=0.121, which is between the μ's at which fractions A and B precipitate (7). Consequently, the ethanol fractionation of AE-glutenin was repeated at μ=0.0005 (70% ethanol adjusted to pH 6.6 with 2N NaOH) and at μ=0.251 (70% ethanol-1.44% acetic acid adjusted to pH 6.6 with 2N NaOH). At these respective μ's, 64.7% and 55.0% of AE-glutenin, by weight, remained soluble. SDS-PAGE showed that the distribution of subunits in the resulting fractions was nearly identical to that at μ=0.121 (Fig. 1); at both μ's, the 36,000, 44,600, and 49,400 MW subunits were present only in the soluble fraction. Thus in neutral 70% ethanol the fractionation of glutenin subunits is similar over a wide range of μ's, and it is unlikely that further fractionation in alcohol solutions can be achieved by varying μ. Fractionation by direct extraction of reduced-alkylated glutenin with 70% ethanol at low μ, however, is not desirable, since slightly more high-MW contaminants are soluble under these conditions. Also, traces of insoluble components may be removed by first dissolving the sample in dilute acetic acid.

An attempt was also made to detect albumin- or globulin-like subunits in glutenin. Glutenin was reduced and extracted for 16 hr. with 0.5M NaCl-0.5% 2-mercaptoethanol (3.5 mg. protein per ml.). Only 7.1% of the protein by weight, however, was solubilized; SDS-PAGE revealed this soluble fraction to consist primarily of 44,000 MW subunits, unlike most wheat albumins or globulins, MWs of which are primarily lower (11). It is conceivable, however, that further

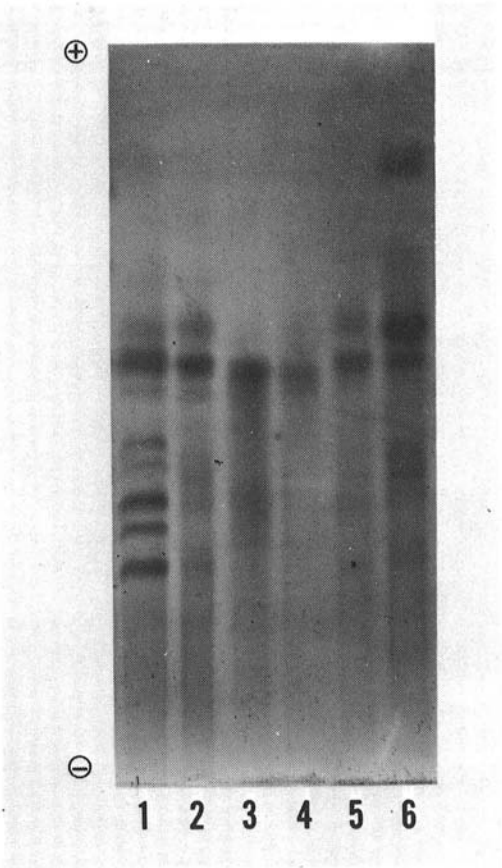

Fig. 4. SDS-PAGE patterns of 1, whole reduced glutenin; 2, ethanol-soluble AE-glutenin; 3, 44,000 MW subunit fraction of ethanol-soluble AE-glutenin; 4, 44,000 MW subunit fraction of high-MW gliadin; 5, high-MW gliadin; and 6, whole gliadin.

fractionation is possible, since Rothfus and Crow (7) and Huebner et al. (10) obtained fractions of glutenin subunits with amino acid compositions unlike glutenin but similar to albumins or globulins.

Comparison of Major Subunits of Soluble AE-Glutenin and High-MW Gliadin

Ethanol-soluble AE-glutenin and reduced high-MW gliadin were each chromatographed on columns of Sephadex G-200, as shown in Fig. 3, and fractions were examined by SDS-PAGE. Before 900 ml., subunits with MWs greater than 44,000 predominate. The major peak in each chromatogram (900–1,300 ml.) contained primarily the 44,000 and 36,000 MW subunits. Fractions underlined in Fig. 3 which contained only 44,000 MW subunits in significant amounts (Fig. 4) were combined; minor contaminants in these fractions from AE-glutenin are apparent only when excess sample is used. To examine heterogeneity within these

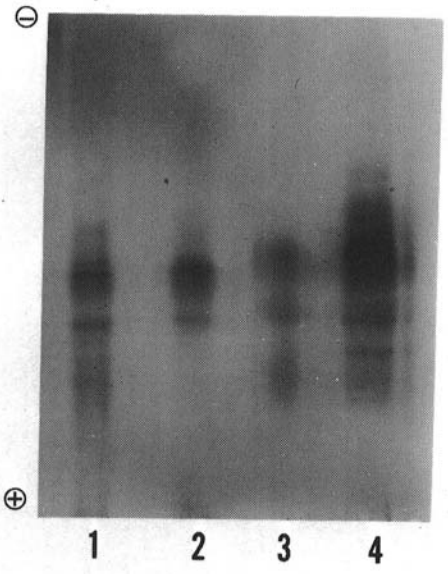

Fig. 5. Starch-gel electrophoretic patterns of 1, PE-high-MW gliadin; 2, PE-derivative of the 44,000 MW subunit fraction of high-MW gliadin; 3, 44,000 MW subunit fraction of ethanol-soluble AE-glutenin; and 4, ethanol-soluble AE-glutenin.

combined fractions, the PE derivatives of the 44,000 MW high-MW gliadin fraction were compared to PE-high-MW gliadin by starch-gel electrophoresis; similarly, the 44,000 MW AE-glutenin subunit fraction was compared to its parent fraction, soluble AE-glutenin. The results, shown in Fig. 5, demonstrate that each 44,000 MW fraction contains fewer electrophoretic subunits than its parent fraction. Several unique polypeptides still occur in each 44,000 MW fraction, however, suggesting that subunit fractions of other MWs may also be heterogeneous.

Amino acid compositions of the 44,000 MW fractions from AE-glutenin and high-MW gliadin are presented in Table II. Compositions are similar but some subunits are certainly unique, as seen from the different proportions of certain amino acids, such as glycine and tyrosine. These compositional data, however, when considered with MWs and charge properties, are still consistent with the concept that some 44,000 MW subunits of high-MW gliadin and soluble AE-glutenin may be closely related.

DISCUSSION

Differential solubility in neutral 70% ethanol has now been used to divide subunits of alkylated-reduced glutenin into two fractions, in a manner analogous to the separation of gliadin from glutenin. Approximately 62% of these glutenin subunits, by weight, are prolamin-like, being soluble in neutral 70% ethanol. MWs

TABLE II. AMINO ACID ANALYSES OF 44,000 MW FRACTIONS ISOLATED FROM SOLUBLE-AE-GLUTENIN AND HIGH-MW GLIADIN[a]

Amino Acid	AE-Glutenin Fraction	High-MW Gliadin Fraction
Lysine	0.4	0.6
Histidine	2.3[b]	1.5
Arginine	1.3	1.9
Aspartic acid	0.9	1.0
Threonine	2.6	2.5
Serine	7.1	7.8
Glutamic acid	41.6	39.6
Proline	15.4	16.2
Glycine	7.4	3.3
Alanine	2.0	2.3
Half-cystine	0.0	1.4
Valine	3.1	4.1
Methionine	0.9	1.4
Isoleucine	2.9	3.6
Leucine	5.6	7.0
Tyrosine	2.6	1.3
Phenylalanine	3.9	4.7

[a]Compositions are expressed as mol. %.

[b]Includes AE-cysteine.

of these subunits are relatively low and homogeneous, and their amino acid composition is similar to that of high-MW gliadin. The ethanol-insoluble subunits, however, are more heterogeneous in MW, contain high-MW subunits, and have amino acid compositions different from gliadin. Thus, wheat glutenin subunits may be fractionated by differences in solubility in alcohol solutions in the same manner as the subunits of corn, barley, and sorghum glutelins, suggesting that prolamin-like subunits occur in glutelins of all cereals.

Crow and Rothfus (6) and Huebner and Rothfus (30) showed that fractions of native glutenin of different MWs gave, after reduction and alkylation, essentially the same starch-gel electrophoretic patterns; thus glutenin probably contains both ethanol-soluble and -insoluble subunits in the same molecule. Simmonds (31,32) has also suggested that glutenin may be derived from several sources, including early formed high-MW proteins (33), lower MW gliadins, various lipoprotein interaction products, and degradation products of membranes and cell organelles in the developing and maturing endosperm. Thus, it is conceivable that the two types of subunits we have observed in glutenin may be synthesized by separate mechanisms or in different locations within the endosperm and joined together during maturation. Our studies have not demonstrated significant incorporation of albumins or globulins into glutenin through covalent bonds, although subunit fractions resembling albumins and globulins have been isolated from glutenin (7,10). We have noted, in other experiments, a marked noncovalent association of these proteins. Further studies are necessary to determine the extent and means of incorporation of albumins or globulins into glutenin.

In our studies, a close similarity has been demonstrated between the ethanol-soluble subunits of glutenin and the subunits of high-MW gliadin. Since high-MW gliadin seems to consist primarily of polypeptides linked by disulfides to form dimers and trimers (4,11), its subunits may contain only a limited number of

cysteine residues available for formation of intersubunit bonds; similarly, the ethanol-soluble subunits of glutenin, such as those of 44,000 MW, may also have limited potential for the formation of intermolecular disulfide bonds, and may have special significance in the structure of glutenin.

The high-MW polypeptides of glutenin, which are the major ethanol-insoluble subunits, are relatively deficient in cysteine (7,10); nevertheless, at least five cysteine residues are present per 133,000 MW subunit (10), giving these polypeptides the potential to combine with low-MW subunits while also forming an extended network through intermolecular disulfide bonding with each other. It is conceivable that the amounts of ethanol-soluble and -insoluble glutenin subunits could strongly influence extent of cross-linking as well as size of the resulting molecule, thereby affecting its functional properties and possibly quality. Further experiments will be necessary to investigate these relationships.

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