Wheat Glutenin Subunits. II. Compositional Differences [1]

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ABSTRACT

Isolation of subunits from reduced wheat glutenin protein by new procedures has permitted detailed chemical and physical studies of them. The subunits were separated from *S*-pyridylethyl derivatives of reduced glutenin by gel filtration into three distinct groups (A, B, and C) which were resolved further by ion-exchange chromatography. Toluenesulfonic acid hydrolysates of the isolated subunits were analyzed for amino acids by modified techniques of automated ion-exchange chromatography. Proteins from fraction A contained considerably more basic amino acids, as well as aspartic acid, than proteins in fractions B and C. Much lower amounts of glutamic acid and proline were present in fraction A proteins than either the B and C fraction or gliadin proteins. Some protein from group B contained the highest amount of glycine–almost six times more than either fraction C or the gliadins. Also, fraction B proteins were lower in valine, isoleucine, and phenylalanine than either A or C proteins. Fraction C proteins resemble gliadins in amino acid composition, except for a slightly higher content of cysteine. Aspartic and glutamic acids were major N-terminal groups of glutenin subunits from all fractions.

The origin and structure of glutenin, the saline- and 70% ethanol-insoluble but acetic acid-soluble high-molecular-weight (MW) protein of gluten, have been widely debated. Evidence has been offered that some polypeptides of glutenin, linked together by intermolecular disulfide bonds, are similar to polypeptides constituting gliadin. Amino acid analyses of both proteins are similar (1,2) and peptide maps of partial enzymatic hydrolyses of gliadin and glutenin contain some peptide components in common (3,4). These findings may have prompted some workers to conclude that glutenin was not a unique protein but an artifact resulting from disulfide interchange of gliadin components during mixing of doughs (5). However,

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the presence of glutenin was confirmed in flours extracted under mild conditions (6) and in early stages of grain development (7).

Considerable heterogeneity has been demonstrated among subunits of reduced glutenin. Differences in MWs of the subunits were established by gel-filtration chromatography (8) and sodium dodecyl sulfate (SDS) electrophoresis (9). Variations in compositions of reduced glutenin fractions obtained from partial separation by precipitation with salts (10) and by isoelectric focusing (11) indicate that glutenin may be composed of numerous polypeptides of different types, some very unlike gliadin. Compositional and physical data on individual polypeptide subunits are essential to understand the structure and properties of glutenins.

The extreme complexity of the mixture of polypeptides that is obtained after reductive cleavage of disulfide bonds proved an obstacle to earlier separation efforts. Using gel filtration, we found that a preliminary separation into groups of proteins differing in MW gives fractions which respond well to further separation procedures (12). Using these techniques, we have isolated fractions much simpler in composition and some that may be individual components. SDS-gel electrophoresis has provided us with a good estimate of their MWs and purity.

We now report the amino acid composition and N-terminal amino acids in various fractions and in purified polypeptides from reduced glutenin isolated by those procedures. These data are compared to those of isolated gliadins and other wheat proteins.

MATERIALS AND METHODS

Source and Composition of Proteins

Protein samples were prepared by fractionation of *S*-pyridylethyl (PE) derivatives of reduced glutenin on Sephadex G-200 columns, followed by ion-exchange chromatography of the fractions on sulfoethyl cellulose (SEC) columns as described earlier (12). All designations of protein fractions are those used in previous separation studies (12). Proteins designated A, B, or C refer to the fractions separated on Sephadex columns, and numerical subgroup designations, such as A-1, refer to components resolved by subsequent chromatography of the fractions on an SEC or another Sephadex column. HC refers to the higher-MW C fraction (44,000) as given in Fig. 7 of ref. 12.

Nitrogen determinations on the fractions were by micro-Kjeldahl procedures. For small samples ammonia in the Kjeldahl digest was determined by a modification of a colorimetric procedure involving a Technicon AutoAnalyzer (13).

Carbohydrate analyses were by the phenol-sulfuric acid method (14).

Amino Acid Analyses

Proteins were hydrolyzed according to the method of Liu and Chang (15) with a 3M aqueous solution of *p*-toluenesulfonic acid (*p*-TSA) containing 0.2% 3-(2-aminoethyl) indole to permit quantitative recovery of tryptophan. Samples were hydrolyzed for 24 and 72 hr. Duplicate amino acid analyses were conducted directly on both hydrolysate solutions with a Beckman automatic amino acid analyzer (16). The short column of Beckman PA-35 resin was increased to 8 cm. to resolve tryptophan and *S*-(4-pyridylethyl)cysteine (PEC) from other basic amino acids. All the cystine in the proteins was converted to cysteine and alkylated to yield PEC, which eluted between ammonia and arginine.

L-α-Amino-β-guanidino-propionic acid (AGPA) (Calbiochem) served as the internal standard for the short column. Generally, the pH 5.28 buffer (Pierce Chemical) had to be made slightly less acidic (pH 5.32) to ensure that PEC would elute between AGPA and arginine. Standard PEC was provided by J. F. Cavins of the Northern Laboratory. Norleucine was the internal standard for the long column.

An Infotronics integrator and an IBM 1130 computer provided automatic computation of amino acid analyses (17).

N-Terminal Amino Acid Analyses

To establish N-terminal residues on the polypeptide chains the proteins were converted to dansyl derivatives and then the dansyl amino acids were isolated from hydrolysates by thin-layer chromatography (TLC). The procedure followed for dansylation, purification, and hydrolysis of proteins was essentially that of Zanetta et al. (18). 1-Dimethylaminonaphthalene-5-sulfonyl chloride (dansyl-Cl) was purchased from Pierce Chemical and the dansylated amino acid standards from Mann Research Labs. Inc. Protein samples ranged from 1.5 to 2.0 mg. Silica gel plates for TLC, 20 × 20 cm., precoated without fluorescence indicator, were from E. Merck A. G. (Darmstadt, Germany). The two-dimensional separation was carried out on glass plates with toluene:pyridine:acetic acid (150:50:3.5) as the first solvent and toluene:chloro-2-ethanol:25% ammonia (100:80:6.7) as the second solvent (18). Reagents for the solvents were analytical grade and used as purchased.

RESULTS

Purity of the Protein Fractions

Carbohydrate analyses of B and C fractions that were resolved on SEC columns indicated the presence of less than 2% carbohydrates. Fractions from peak A, which were further separated by passage through a column of Sephadex G-200 with 6M GHCl as solvent, analyzed up to 20% carbohydrate. It is likely that most of the carbohydrate was derived from the Sephadex column (19). By redissolving the fractions in dilute acetic acid and precipitating with 5 to 10% trichloroacetic acid, the protein sample was purified to less than 7% carbohydate.

Amino Acid Analyses

Calculations for amino acid composition incorporated corrections for destruction of amino acids or low recoveries. Destruction of threonine and serine with time during hydrolysis was similar to that observed by Liu and Chang (15) for other proteins with both HCl and *p*-TSA. Results of linear extrapolation of 24- and 72-hr. values of these amino acids to zero time are given in Table I for two protein fractions. Since recoveries for valine and isoleucine were uniformly higher at 72 hr., these values were selected. Also as noted in Table I, both leucine and tyrosine gave slightly higher recoveries at 72 hr. so their analyses were adjusted to the 72-hr. values. While Liu and Chang (15) found that cystine degraded with time during hydrolysis, we observed that PEC analysis gave good duplication at both hydrolysis times. These findings agree with those of Cavins and Friedman (20) that PEC is highly stable to acid hydrolysis and that prior derivatization of cysteine with vinylpyridine improves its quantitative recovery. Since tryptophan values varied more, some low values may indicate significant destruction due to the presence of carbohydrate. The presence of small amounts of residual urea or GHCl in some

samples caused the amino acid recovery based on sample weight or nitrogen to be low. Therefore, the amino acid analyses were adjusted to 98% total amino acid recovery to allow for possible small amounts not recovered after hydrolysis.

TABLE I. YIELD OF AMINO ACIDS AT DIFFERENT HYDROLYSIS TIMES OF WHEAT GLUTENIN SUBUNITS

	Fraction B-1			Fraction HC-3		
Amino Acid	24 hr.	72 hr.	Calculated value[a]	24 hr.	72 hr.	Calculated value[a]
Threonine	26.6	26.3	26.7	22.3	22.1	22.4
Serine	54.2	51.0	56.0	70.2	63.0	73.7
Valine	13.2	16.2	16.2	33.1	35.6	35.6
Isoleucine	4.5	5.8	5.8	24.7	29.7	29.7
Leucine	38.4	40.4	40.4	59.2	60.8	60.8
Tyrosine	47.3	50.6	50.6	10.0	12.2	12.2

[a]Results for threonine and serine were extrapolated to zero time; 72 hr. was needed for valine, isoleucine, leucine, and tyrosine. All values given in moles per 10^5 g.

Amino Acid Compositions

The amino acid compositions of a number of representative chromatographic fractions of reduced alkylated glutenin polypeptides from each of the groups isolated from the Sephadex column are compared in Table II. Results expressed in percentage amino acids permit relative amounts of different amino acids to be compared easily both within and between subunits. Also, data are given in moles per 100,000 g. protein to permit comparison with results of other workers. The data clearly establish that differences in amino acids occur between all fractions but that the greatest differences exist among those from the different groups A, B, and C isolated from the initial Sephadex column. Figure 1 shows a graph relating the amounts of amino acids which vary significantly between some of the fractions.

One of the most prominent differences in composition is in the fraction A proteins, which contain a higher proportion of basic amino acids than the B or C fractions. Fraction A-1 contained three to four times more lysine than fractions B-1, B-6, or HC-3. Also aspartic acid is several times higher in the A fractions than in any of the others. In contrast, the percentage of glutamic acid and proline is much lower in the A fractions compared to the amounts in any of the B or C fractions.

The B fractions are characterized by an unusually high percentage of glycine, a significantly greater tyrosine content, and a much lower proportion of cystine and phenylalanine. Approximately 72% of fraction B-1 consists of only three amino acids, glutamic acid, proline, and glycine. In contrast, these three amino acids constitute only 41% of fraction A-1 and 56% of HC-3.

Of the C fractions, the composition of only fraction HC-3 is given in Table II, because 24-hr. hydrolysates of other C fractions were all similar in amino acid content. The C fractions were characterized by high glutamic acid and proline contents similar to the B fractions. However, the percentages of leucine, isoleucine, valine, and phenylalanine exceeded that of the B fractions rendering the C fractions less polar.

TABLE II. AMINO ACID COMPOSITION OF FRACTIONS FROM REDUCED AND ALKYLATED PONCA WHEAT GLUTENIN[a]

Amino Acid	A-1		A-2		B-1		B-2		B-6		HC-3		Whole PE-Glu[b]	
Tryptophan	1.2	(10.6)	0.6	(5.2)	0.8	(7.2)	0.9	(8.1)	0.5	(4.4)	0.2	(1.8)	0.5	(4.3)
Lysine	3.3	(29.2)	2.4	(20.8)	0.8	(6.9)	0.9	(8.1)	1.0	(8.7)	0.7	(5.8)	1.5	(12.7)
Histidine	2.0	(17.7)	1.9	(16.5)	0.5	(4.4)	0.6	(5.4)	1.6	(13.6)	1.5	(12.4)	1.6	(13.6)
Arginine	3.8	(33.6)	3.1	(27.0)	1.2	(10.6)	1.5	(13.5)	2.1	(18.0)	1.9	(15.7)	2.4	(20.4)
Aspartic acid	6.8	(60.0)	5.2	(45.2)	0.7	(6.1)	0.7	(6.3)	1.1	(9.6)	1.5	(12.4)	2.7	(23.0)
Threonine	4.1	(36.3)	3.8	(33.0)	2.9	(26.7)	3.2	(28.6)	3.3	(29)	2.7	(22.4)	3.2	(27.2)
Serine	7.0	(62.0)	8.3	(72.2)	6.2	(56)	7.2	(64.8)	7.4	(66.5)	8.9	(73.7)	7.4	(63.2)
Glutamic acid	20.9	(185.0)	25.2	(219)	37.8	(346)	37.2	(336)	36.9	(323)	37.7	(312)	33.3	(283)
Proline	9.3	(82.3)	10.3	(90)	14.4	(132)	12.7	(115)	11.7	(102)	14.9	(123)	13.0	(111)
Glycine	10.6	(93.8)	6.2	(53.8)	19.4	(178)	19.2	(174)	14.2	(124)	3.6	(29.7)	8.4	(71.5)
Alanine	6.5	(57.5)	5.2	(45.2)	2.0	(18.8)	2.4	(21.5)	2.6	(22.7)	2.3	(19.0)	3.4	(29.0)
Half-cystine[c]	0.9	(8.0)	1.7	(14.8)	0.4	(3.9)	0.5	(4.5)	1.3	(11.4)	2.2	(18.2)	1.8	(15.3)
Valine	5.4	(47.8)	5.6	(48.7)	1.8	(16.2)	1.8	(16.0)	2.8	(24.5)	4.3	(35.6)	4.0	(34.0)
Methionine	1.7	(15.1)	1.8	(15.7)	0.6	(5.5)	0.5	(4.5)	0.9	(7.9)	1.7	(14.0)	1.4	(11.9)
Isoleucine	3.6	(31.8)	3.8	(33)	0.6	(5.8)	0.7	(6.3)	2.0	(17.5)	3.6	(29.7)	3.0	(25.5)
Leucine	3.8	(33.6)	8.0	(69.5)	4.3	(40.4)	4.1	(37.0)	5.0	(43)	7.3	(60.5)	6.5	(55.4)
Tyrosine	3.9	(34.5)	2.9	(25.2)	5.4	(50.6)	5.8	(52.3)	4.4	(38)	1.4	(12.2)	3.0	(25.5)
Phenylalanine	3.6	(31.8)	4.5	(39)	0.4	(3.6)	0.5	(4.5)	1.6	(14)	4.6	(38.0)	3.6	(30.6)

[a]Values shown are as molar percent of the total protein; values in parentheses are moles per 10^5 g. protein. Fractions are results of separation described in reference 12, Figs. 4, 7, and 8.

[b]PE-Glu = *S*-pyridylethyl-glutenin.

[c]Although cysteine was analyzed as *S*-(4-pyridylethyl)cysteine (see Methods), it was left in its usual order to avoid confusion.

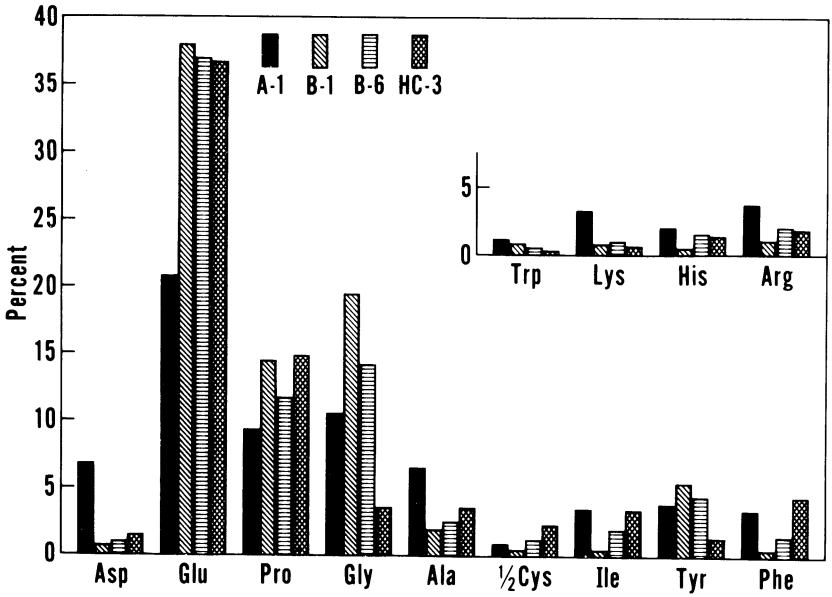

Fig. 1. Percentages of some amino acids from four fractions A-1, B-1, B-6, and HC-3, taken from Table 1, that show the major variations among fractions of wheat glutenin subunits.

Comparison of Glutenin Subunits with Other Wheat Proteins

Amino acid compositions of various classes of wheat proteins have been given by Woychik et al. (21). In comparing amino acid composition of the glutenin fractions with those of different wheat proteins, the composition of fraction A appears to be unlike the amino acid composition of any of them except for water-solubles consisting of albumins and globulins. As shown in Table II, the fraction A proteins, like albumins and globulins, are lower in glutamic acid and proline and richer in basic amino acids than are the other wheat proteins.

The C group of polypeptides, including the quite pure HC-3 fraction, is similar to gliadin proteins in amino acid composition and MWs. SDS electrophoresis (9) has enabled us to estimate the MW of the polypeptides in our previous study (12). Therefore, it is possible to calculate the approximate number of amino acid residues per molecule for two of the purer polypeptides. These data are given in Table III for polypeptides B-1 and HC-3. The number of amino acid residues in some gliadin proteins previously purified is also given (22–24). Apparently the HC-3 composition most closely corresponds to the high-MW gliadin fraction isolated by Beckwith et al. (24).

The B fractions of glutenin not only have high MW, but amino acid compositions that do not correspond to other proteins of wheat. The HC-3 fraction has much more cysteine than the B-1 although the latter molecule is over three times as large as the former. Also, there is less phenylalanine and isoleucine in the larger molecule of B-1 than in HC-3.

N-Terminal Amino Acids

Table IV lists the results of N-terminal amino acid analysis. Dansyl derivatives of

TABLE III. RESIDUES OF AMINO ACIDS PER MOLE

Amino Acid	PE-Glu Subunits		Gliadin Proteins		High-MW Gliadin
	B-1	HC-3	γ_3 (22)	α_2 (23)	(24)
Tryptophan	10	1	2	1	...
Lysine	10	3	2	1	2
Histidine	6	5	4	5	5.6
Arginine	14	7	4	5	6
Aspartic acid	8	5	6	8	5.2
Threonine	36	10	7	4	8.8
Serine	75	32	14	14	24.4
Glutamic acid	461	136	124	104	130
Proline	176	54	59	39	53
Glycine	236	13	8	7	12
Alanine	25	8	10	7	8.8
Half-cystine	5	8	6	5	4
Valine	22	16	13	12	15.6
Methionine	7	6	4	3	5.2
Isoleucine	8	13	14	12	12.4
Leucine	53	27	20	21	25.2
Tyrosine	65	5	2	8	4.8
Phenylalanine	5	17	18	10	15.6
Ammonia	460	138	130	95	126
MW	130,600	41,740	36,500	31,050	40,000
Total residues	1,222	366	317	266	...

TABLE IV. N-TERMINAL AMINO ACIDS OF SOME PONCA PE-GLU FRACTIONS

Samples[a]	Identified on TLC	
	Strong	Weak
A-2	Aspartic acid, glutamic acid	
B-1	Glutamic acid	
B-5	Aspartic acid, glutamic acid	
B-6	Aspartic acid, glutamic acid	
HC-3	Aspartic acid	Glutamic acid, isoleucine
HC-4	Aspartic acid, threonine	Glutamic acid, tryptophan, isoleucine, tyrosine
HC-5	Aspartic acid, glutamic acid	Threonine

[a]Samples from Figs. 4, 7, and 8, reference 12.

either aspartic acid, glutamic acid, or both, were obtained from all the glutenin fractions, and they appear to be the main N-terminal amino acids. Isoleucine, phenylalanine, proline, threonine, and serine were found as dansyl derivatives in trace amounts on the TLC from nearly all the samples. This observation could indicate that the method is not completely specific for end groups. Some weak unidentified spots may have been due to small peptides, which migrated close to the position of some amino acids. Peptide formation is possible because hydrolysis had to be conducted under mild conditions to prevent destruction of dansyl-amino acids. Threonine and serine dansyl derivatives were difficult to separate by TLC. Solvent systems reported to separate these two amino acids (25) were tried without success. Generally, threonine seemed to be the amino acid present when a spot occurred in the region of their migration.

DISCUSSION

The main conclusion resulting from analysis of these highly resolved fractions of pyridylethyl glutenin polypeptides is that each of the three major groups A, B, and C contains proteins which differ significantly from the other groups in their amino acid content. Also, the chromatographic separations of these groups resolved numerous components establishing that reduced glutenin composition is even more complex than either starch-gel (26) or SDS electrophoresis (9) indicates.While some differences in amino acid composition occur between proteins belonging to each of these groups, the differences are relatively small compared to the differences between groups. These variations in composition of the major groups of reduced glutenin probably account for conflicting ideas in the earlier literature about the nature of glutenin subunits.

While the amino acid content of the A fractions more closely resembles that of albumins than that of other wheat proteins, the solubility characteristics and MWs of these two protein groups differ. Aggregation of group A proteins occurs even in high concentrations of urea in contrast to the albumins which are readily dissociated in aqueous media. Also the A-1 and A-2 fractions show a wide range of MWs in contrast to the wheat albumins which generally are of low MW (10,000 to 20,000). Only the A-3 fraction shows low MWs similar to water-soluble proteins (12). Other workers have suggested that albumins or globulins may be present in glutenin, and this conclusion may be based on some similarities to the A-group proteins, especially the A-3 fraction. Additional work will be required to determine whether ionic, hydrophobic, or other bonds are responsible for the tendency of A-group proteins to associate.

Fraction B proteins appear unique to glutenin, exhibiting greater differences in amino acid composition compared to other wheat proteins than either the A or C fractions. The high proportions of glycine, proline, and glutamic acid in hydrolysates of B polypeptides suggest a possible structural role for these proteins. Glycine and proline are major constituents of collagen and other fibrous proteins (27). The high MWs of these B chains are also consistent with their structural roles. The low content of half-cystine residues in the B subunits would not allow for many intramolecular disulfide bonds, thereby favoring a fairly linear chain with few cross-links to other subunits.

The fraction C proteins resemble gliadin proteins in many properties, including amino acid composition and MW. Most gliadin molecules possess only intramolecular disulfide bonds in contrast to glutenin, which appears to be held together by interchain disulfide groups. However, the fraction C polypeptides appear most closely related to polypeptides that make up the high-MW fraction of gliadin in which several chains are linked by disulfide bonds. Evidently these proteins must differ from ordinary gliadin by having structures favoring formation of intermolecular disulfides as shown by Beckwith and Wall (28). The higher quantity of cysteine in fraction C compared to gliadin may contribute to this tendency.

The present finding agrees with other recent work showing major differences among the glutenin subunits. Mita and Yonezawa (11) used isoelectric focusing to obtain an insoluble fraction and several soluble fractions of cyanoethylated glutenin. Their insoluble C fraction closely resembles our A fractions in amino acid content, solubility characteristics, and starch-gel electrophoretic behavior. However,

their fractions I and III, for which amino acid data are also presented (11), appear intermediate between our B and C fractions in composition. Resolution of B and C fractions, which differ mainly in content of glycine and nonpolar amino acids, is possibly not well achieved by isoelectric focusing alone. On the other hand, Rothfus and Crow (10) used increasing concentrations of $Cu(NO_3)_2$ to fractionate aminoethyl glutenin and at their lowest level of $Cu(NO_3)_2$ (0.0283M) precipitated a fraction rich in glycine and low in nonpolar amino acids. This fraction closely resembles our fraction B in composition and properties. However, our chromatographic methods yield far greater separation than the procedures described by previous workers.

The polypeptide fractions, which we analyzed for N-terminal amino acids, appeared to contain either aspartic or glutamic acids in that position. Mita and Yonezawa (11) found that three of their fractions contained glutamic acid as the major N-terminal amino acid, but fractions P, I, and III, which seemed most pure, contained alanine, phenylalanine, and glycine, respectively, as the N-terminal group. The reason for these differences from our results is not known. The large number of residues containing N-terminal aspartic acid suggests that many glutenin proteins may have common N-terminal sequences.

The considerable variation between the composition of the three major fractions of glutenin polypeptides raises questions as to the origin of glutenin. Some authors speculate that glutenin is formed by the interaction of proteins from the protein bodies with reticular matrix and soluble proteins (29). Further studies are required to establish whether the different types of glutenin proteins are all linked together by disulfide bonds or whether only like types are disulfide-linked and can be separated physically.

Preliminary gel-filtration results with reduced glutenins from other varieties indicate there may be different percentages of the three fractions A, B, and C. This variation may be responsible for differences in types and amounts of subunits in reduced glutenin from varieties of wheats as observed by starch-gel (26) and SDS electrophoresis (9). It may also be the reason why native glutenins from varieties differing in baking quality exhibit different ranges of MW distribution (30). Such variation in composition and physical properties of glutenins from different wheats may be related to their characteristics that influence baking and mixing quality of doughs.

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