# Studies of Glutenin. XIII. Gel Filtration, Isoelectric Focusing, and Amino Acid Composition Studies[1]

K. KHAN and W. BUSHUK, Department of Plant Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

ABSTRACT

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Glutenin, isolated from the hard red spring wheat cultivar Manitou, was reduced with β-mercaptoethanol and alkylated with 4-vinylpyridine. Gel-filtration chromatography on Sephadex G-200 was done with four different aqueous solvents (acetic acid, acetic acid-urea [AU], acetic acid-urea-hexadecyltrimethylammonium bromide, and tris-HCl-sodium dodecyl sulfate). Each solvent produced an elution profile with three peaks; the amount of protein represented by each peak depended on the solvent. In all cases, peak I eluted with the void volume (mol wt > 200,000). The three peaks obtained with each solvent were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed qualitative and quantitative differences among the peaks. The three obtained with the AU solvent were, however, chosen for further detailed characterization since SDS-PAGE showed that each peak obtained with this solvent comprised subunits of different molecular weights. Peak I protein comprised subunits with molecular weights ranging from 12,000 to 68,000. Peak II contained the highest molecular weight subunits, ranging from 68,000 to 134,000. Peak III proteins contained predominantly the 35,000 and 45,000 mol wt subunits. Amino acid compositions of the three peaks were markedly different especially in the proportions of basic, acidic, and hydrophobic amino acids. Peak II proteins were examined by isoelectric focusing on a 6–8 pH gradient of Ampholine® carrier ampholytes in 6*M* urea. Elution of the focused proteins gave a profile with 12 peaks (fractions). These 12 fractions contained 20 different protein species, detected by SDS-PAGE. Two homogeneous subunits, 134,000 and 90,000 mol wt, were isolated by isoelectric focusing. The two subunits were partially characterized; they differed in amino acid composition and N-terminal amino acid.

The importance of glutenin in bread-making quality has been confirmed through fractionation (Orth and Bushuk 1972, Orth et al 1972, Pomeranz 1965, Shogren et al 1969) and reconstitution (baking) studies (Shogren et al 1969). Physicochemical studies such as salt fractionation (Rothfus and Crow 1968), gel filtration (Arakawa and Yonezawa 1975; Arakawa et al 1977; Hamauzu et al 1975; Huebner and Wall 1974, 1976; Meredith and Wren 1966), ion-exchange chromatography (Huebner and Wall 1974), isoelectric focusing (Mita and Yonezawa 1971), fractionation by pH precipitation (Danno et al 1978), and amino acid composition and N-terminal amino acid analysis (Arakawa et al 1977, Huebner et al 1974, Mita and Yonezawa 1971) have been used in an attempt to elucidate the properties of glutenin that may be important in bread-making quality. Those studies have revealed many unique features of glutenin in physical properties and chemical composition that may indeed be relevant to the functionality of this protein. The exact relationships between structure and function remain to be discovered, however.

This article reports additional information on glutenin, based on gel filtration, isoelectric focusing, and salt fractionation, that may be useful in resolving the complex structure of this extremely important protein.

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## MATERIALS AND METHODS

### Extraction and Alkylation of Glutenin

Glutenin was isolated from flour milled from the hard red spring wheat cv. Manitou on a Buhler pneumatic experimental mill and purified with SP Sephadex (Pharmacia, Uppsala, Sweden) according to the procedure of Orth and Bushuk (1973a). The purified freeze-dried glutenin was reduced with β-mercaptoethanol, and alkylated with 4-vinylpyridine according to the method of Friedman et al (1970).

### SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at pH 7.3 according to Orth and Bushuk (1973b) and at pH 8.4 according to a modified procedure of Khan and Bushuk (1977). In their previous study, Khan and Bushuk (1977) used 0.125*M* tris, the pH of which was adjusted to 8.9 with boric acid (0.03*M*) to give the tris-borate buffer. Use of this buffer with a higher boric acid concentration (0.125*M*) and lower pH (8.4) was later found to give improved resolution (sharper protein bands) in the lower molecular weight region. The pH 8.4 system was therefore adopted for routine SDS-PAGE analysis used in this study.

Staining and destaining of gels were done according to the procedure of Khan and Bushuk (1977).

### Gel-Filtration Chromatography

The gel-filtration columns were prepared as follows: Sephadex G-200 was dispersed in distilled water, heated in a boiling water bath for 5 hr to facilitate hydration, cooled to room temperature, and equilibrated with appropriate elution solvent. The eluants used for gel filtration were 0.1*N* acetic acid, 0.1*N* acetic acid-3*M* urea (AU), 0.1*N* acetic acid-3*M* urea-0.01*M* hexadecyltrimethylammonium bromide (AUC), and 0.1*M* tris-HCl containing 0.1% (w/v) SDS. The Sephadex slurry was deaerated and poured into a 2.5 × 100-cm or a 5.0 × 100-cm column as described in the booklet "Gel-filtration in Theory and Practice" (Pharmacia Fine Chemicals, Uppsala, Sweden). Sodium azide (0.02%) was included in the equilibrating buffer to prevent microbial growth. The downward flow rate was controlled by a Mariotte-type container (see above-mentioned booklet). The eluate was monitored continuously on an ultraviolet recorder and fractions were collected with an automatic fraction collector. Appropriate fractions were pooled, dialyzed against distilled water, and freeze-dried. Protein losses in the gel-filtration fractionations amounted to less than 5%.

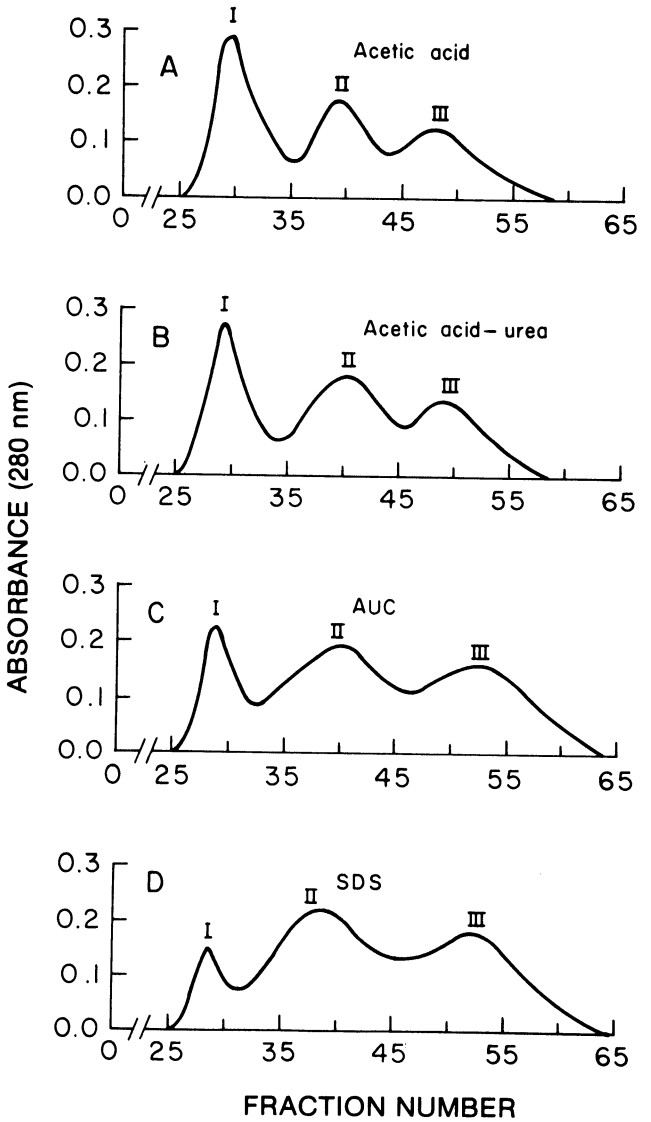


**Fig. 1.** Gel-filtration profiles of RA-glutenin (cv. Manitou) on Sephadex G-200 with four different solvents. Column, 2.5 × 100 cm; bed height, 80 cm; sample concentration, 12.5 mg/ml; flow rate, 20 ml/hr; AUC = acetic acid-urea-hexadecyltrimethylammonium bromide; SDS = tris-HCl-sodium dodecyl sulfate.

### Ammonium Sulfate Fractionation

Solid ammonium sulfate was added slowly to reduced alkylated (RA-) glutenin dissolved in AUC solvent. The suspension was left at 2°C for 2 hr and then centrifuged at 20,000 × *g* for 30 min to give a supernatant and a precipitate, both of which were dialyzed against distilled water and freeze-dried. Some experiments were repeated three times; results of these showed that the precipitation procedure was reproducible. Protein losses in this fractionation amounted to less than 8%.

### Isoelectric Focusing

The isoelectric focusing (electrofocusing) procedure used was based on that of Jamieson et al (1972). A 220-ml focusing column (similar to the LKB 8100-1 column) and a gradient mixing device obtained from Kontes Glass Company, Vineland, NJ were used. Experiments were performed with Ampholine carrier ampholytes (LKB) with the pH ranges 3–10, 5–9, and 6–8.

The column was prepared as follows. The dense electrode solution, comprising 0.4 ml concentrated sulfuric acid, 24 g sucrose, and 28 ml of 6*M* urea solution, was added to the anode at the bottom of the column. Sucrose solution, containing the carrier ampholytes, was introduced slowly into the column using the density gradient mixer to form the required gradient. The dense gradient solution comprised 56 g sucrose, 3.6 ml carrier ampholytes, and 84 ml of urea solution. The light gradient solution (LGS) contained 1.2 ml carrier ampholytes in 120 ml of 6*M* urea solution. The final concentration of carrier ampholytes in the column gradient was approximately 1% (w/v). The glutenin (40–50 mg) was dissolved in a small volume (about 10 ml) of LGS. This solution was added to the LGS when about 25 ml of the LGS had entered the column. When the column was filled, the light electrode solution (10 to 20 ml of 1% [w/v] NaOH solution) was added to the cathode (top of the column). About 4 to 5 hr was required to fill the column. All operations were performed at 2°C with water at 2°C circulating through the cooling jackets of the column. When focusing was completed, the valve at the bottom of the column was closed and the column was emptied through the lower exit at a flow of about 0.5 ml/min. Fractions of about 1.5 ml were collected with an automatic fraction collector. The pH and absorbance at 280 nm of the fractions were measured. Appropriate fractions were pooled, dialyzed against distilled water, and freeze-dried for subsequent analyses.

### Carbohydrate Determination

Carbohydrate content of peak I, II, and III fractions, from gel filtration, was determined by the anthrone method according to Spiro (1966). Glucose was used as the standard sugar.

### N-Terminal Amino Acid Determination

The procedure used for determining N-terminal amino acid was that of Zanetta et al (1970). The N-terminal amino acid was converted to the dansyl derivative by reaction with 1-dimethylaminonaphthalene-5-sulfonyl chloride (dans-Cl) and then hydrolyzed. Dans-Cl and dans-amino acid standards were purchased from Pierce Chemical Company. Glutenin samples used for dansylation ranged from 1 to 2 mg.

Thin-layer chromatography, used to identify the dansyl amino

acids, was performed on polyamide sheets (5 × 5 cm, Cheng-Chin or Schliecher and Schuell). The solvent used was either benzene/acetic acid (9:1) or formic acid/water (1.5:100).

### Amino Acid Composition

Amino acid compositions were determined on a Beckman Model 121 automatic amino acid analyzer. Protein samples were prepared for analysis according to the procedure of Orth et al (1974). Values for ammonia are not reported because they are affected by nitrogen containing substances such as urea used in extraction of glutenin in gel filtration, and from carrier ampholytes used in electrofocusing.

### Reagents

Urea solutions were purified by passing through a bed of mixed resin (Bio Rad AG 501-X8 (D), 20–50 mesh). 4-Vinylpyridine (Aldrich Chem. No. 320-4) was purified by distillation before use. All other chemicals were of reagent grade.

## RESULTS AND DISCUSSION

### Gel-Filtration Chromatography

A number of reports (Arakawa and Yonezawa 1975, Arakawa et al 1977, Hamauzu et al 1975, Huebner and Wall 1974) showed that gel filtration on Sephadex G-100, G-150, and G-200 of reduced alkylated glutenin (RA-glutenin) in acetic acid and acetic acid-urea solvents produced three peaks (fractions) with peak I eluting in the void volume. Upon SDS-PAGE, however, peak I proteins contained subunits with molecular weights ranging from 68,000 to 12,000. Peak I proteins, therefore, exhibited unusually strong aggregating properties. In the present study four solvents, differing in their disaggregating (dissociating) properties, were used in gel filtration of RA-glutenin to study the tendency to aggregate of peak I proteins. The four solvents used were 0.1 *N* acetic acid, 0.1 *N* acetic acid-3 *M* urea (AU), 0.1 *N* acetic acid-3 *M* urea-0.01 *M* hexadecyltrimethylammonium bromide (AUC), and 0.1 *M* tris-HCl-0.1% (w/v) SDS, pH 8.4 (SDS).

The elution profile obtained with each solvent showed three peaks with peak I eluting in the void volume (Fig. 1). However, the amount of protein in peak I decreased as the dissociating ability of the solvent increased (Table I). The four solvents ranked in the following order in their ability to dissociate peak I glutenin aggregates: SDS > AUC > AU > acetic acid. As the dissociating ability of each solvent increased, the amount of protein in peaks II and III increased (Table I).

Figure 2 shows the SDS-PAGE patterns of the three peaks obtained with the four solvents and the unfractionated glutenin. The patterns of peak I proteins (patterns A,D,G,J) differ qualitatively and quantitatively. Fewer and less intensely stained protein bands in patterns are obtained with AUC and SDS solvents (patterns G,J). Also, for these two solvents, more subunits in the 68,000 to 12,000 mol wt region were observed for peaks II (H,K) and III (I,L). These observations are consistent with the quantitative results of the gel-filtration experiments (Table I). Since SDS-containing solvent dissociated a large proportion of peak I proteins, the tendency of peak I proteins to aggregate probably is due, primarily, to hydrophobic interactions (SDS disrupts such interactions in protein solutions).

Because the objective of gel filtration was to fractionate RA-glutenin into groups of fewer subunits to facilitate further purification and characterization, the aggregating tendency of peak I subunits was used to advantage for this purpose. Accordingly, for preparative gel filtration, Sephadex G-200 and AU solvent were used. Urea was incorporated as a hydrogen bond-breaking agent to minimize association through hydrogen bonding.

With the AU solvent, RA-glutenin fractionated (on Sephadex G-200) into three different groups of subunits, a very convenient result for the purposes of the present study. The subunit molecular weight

**TABLE I**
**Quantitation of Protein[a] Fractions of Reduced Alkylated (RA-) Glutenin[b] (cv. Manitou) from Gel Filtration on Sephadex G-200 with Various Solvents**

| Solvent | Protein (% Recovered) | | |
|---|---|---|---|
| | Peak I | Peak II | Peak III |
| Acetic acid | 22 | 33 | 40 |
| Acetic acid-urea | 21 | 34 | 40 |
| Acetic acid-urea-hexadecyltrimethylammonium bromide | 17 | 38 | 42 |
| Sodium dodecyl sulfate | 8 | 45 | 43 |

[a] Determined by micro-Kjeldahl technique.
[b] Concentration of glutenin applied to Sephadex column was 12.5 mg/ml.

**TABLE II**
**Fractionation of RA-Glutenin with Ammonium Sulfate at Two (2.5 and 12.5 mg/ml) Initial Protein Concentrations**

| Ammonium Sulfate (mg/ml) | Supernatant | | Precipitate | |
|---|---|---|---|---|
| | 2.5 (%) | 12.5 (%) | 2.5 (%) | 12.5 (%) |
| 16 | 92 | 84 | 2 | 8 |
| 33 | 88 | 76 | 4 | 16 |
| 66 | 80 | 64 | 12 | 30 |
| 132 | 56 | 22 | 40 | 72 |
| 200 | 28 ($S_1$)[a] | ... | 69 ($P_1$)[a] | ... |

[a] Fractions used in subsequent experiments.

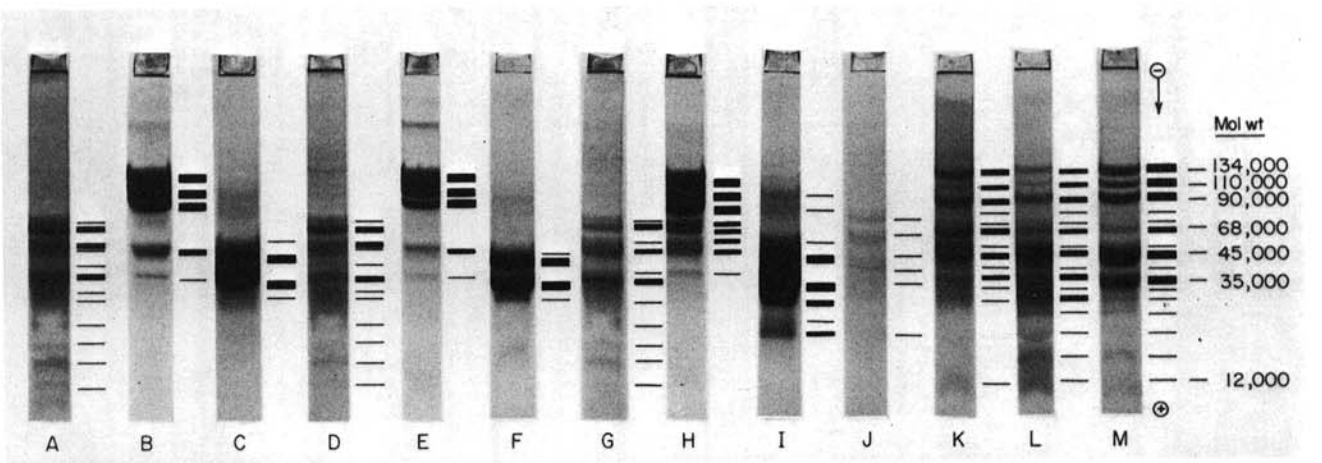


**Fig. 2.** SDS-PAGE at pH 7.3 of gel-filtration fractions of RA-glutenin as in Fig. 1: **A–C,** peaks I–III, acetic acid; **D–F,** peaks I–III, acetic acid-urea; **G–I,** peaks I–III, AUC; **J–L,** peaks I–III, SDS; and **M,** RA-glutenin.

distribution of the three fractions (Fig. 2D–F) was as follows: peak I, 68,000 to 12,000; peak II, 68,000 to 134,000; and peak III, predominantly 35,000 and 45,000. With the AUC and SDS solvents, peaks II and III contained substantial amounts of lower molecular weight (68,000–12,000) subunits; this complicates further purification.

**Ammonium Sulfate Fractionation**

Fractionation by precipitation with ammonium sulfate of RA-glutenin solubilized in AUC was done to determine if the subunits that eluted in the void volume on Sephadex G-200 (peak I) could be precipitated at a lower salt concentration than that required for the higher molecular weight subunits of peak II. A series of precipitations, with different concentrations of ammonium sulfate, were performed at two concentrations of RA-glutenin, 2.5 and 12.5 mg/ml (Table II). The concentration of 12.5 mg/ml was chosen because that concentration of protein was applied to the Sephadex columns in gel-filtration experiments.

Table II shows that, at both initial glutenin concentrations, the amount of protein in the supernatant decreased but that in the precipitate fractions increased as the concentration of ammonium sulfate increased. At the lower glutenin concentration (2.5 mg/ml), more protein remained in the supernatant than at the higher concentration at the same ammonium sulfate concentration.

SDS-PAGE patterns of the fractions obtained by ammonium sulfate precipitation at initial glutenin concentrations of 12.5 and 2.5 mg/ml are shown in Figs. 3 and 4, respectively.

With the higher glutenin concentration, the precipitate at 16, 33, and 66 mg/ml of ammonium sulfate (Fig. 3A–C) contained subunits with 68,000–12,000 mol wts. In this respect, these precipitates were similar to the peak I fraction obtained by gel filtration on Sephadex G-200.

In contrast, the patterns of the analogous precipitates (Fig. 4A–C) of the 2.5 mg/ml experiment were essentially the same as the pattern for unfractionated RA-glutenin. These results show that the precipitation of glutenin by ammonium sulfate strongly depends on the initial protein concentration.

When the ammonium sulfate concentration was increased to 132

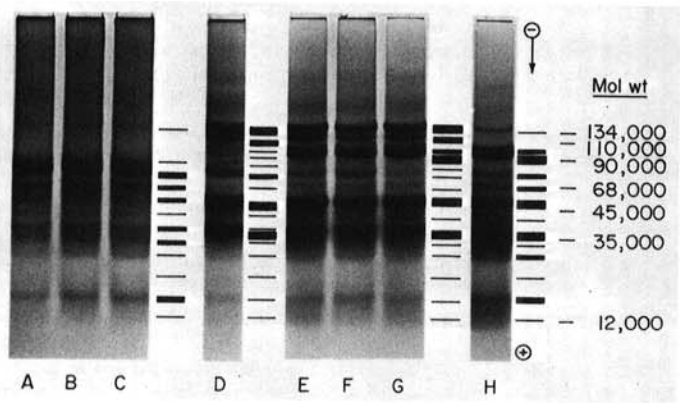


**Fig. 3.** SDS-PAGE at pH 8.4 of fractions obtained from fractionation of RA-glutenin (12.5 mg/ml) with ammonium sulfate: **A–D,** precipitates and **E–H,** supernatants from 16, 33, 66, and 132 mg/ml of ammonium sulfate, respectively.

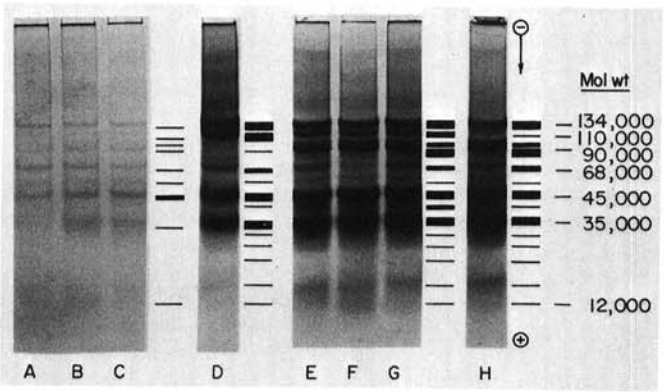


**Fig. 4.** SDS-PAGE at pH 8.4 of fractions obtained from fractionation of RA-glutenin (2.5 mg/ml) with ammonium sulfate: **A–D,** precipitates and **E–H,** supernatants from 16, 33, 66, and 132 mg/ml of ammonium sulfate, respectively.

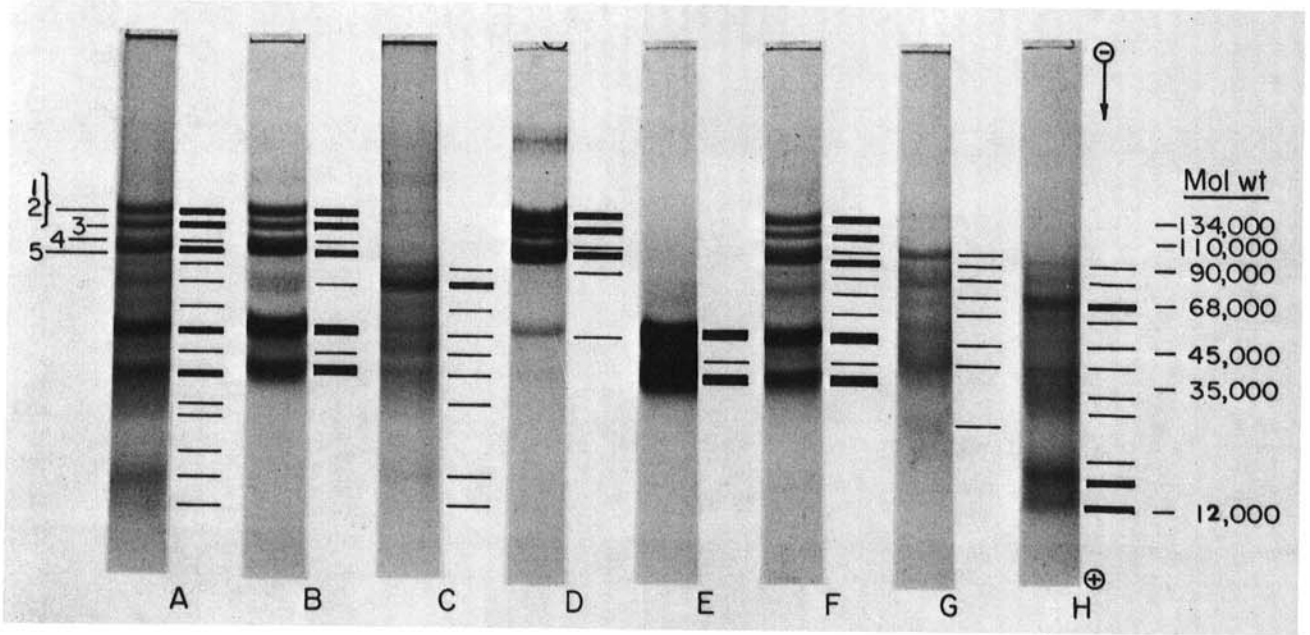


**Fig. 5.** SDS-PAGE at pH 8.4 of fractions from ammonium sulfate fractionation of RA-glutenin (2.5 mg/ml): **A,** RA-glutenin; **B,** $P_1$; **C,** peak I from gel filtration of $P_1$; **D,** peak II from $P_1$; **E,** peak III from $P_1$; **F,** $P_2$ (precipitate from ammonium sulfate fractionation of $P_1$); **G,** $S_2$ (supernatant from ammonium sulfate fractionation of $P_1$); and **H,** $S_1$ (supernatant from ammonium sulfate fractionation of total RA-glutenin). (**1–5** on the left refer to the first five high molecular weight subunits of glutenin.)

mg/ml (or higher), the high molecular weight subunits (90,000–134,000) appeared in the patterns of the precipitates (Figs. 3D and 4D).

The SDS-PAGE patterns of the supernatants for both experiments (Figs. 3E–G and 4E–G) were the same and essentially identical to that for the unfractionated RA-glutenin. However, the supernatant obtained from precipitation of the 12.5 mg/ml of RA-glutenin with 132 mg/ml of ammonium sulfate showed fewer subunits (Fig. 3H) in the 90,000–134,000 mol wt region than the supernatant obtained (Fig. 4H) from precipitation of the 2.5 mg/ml of RA-glutenin at the same ammonium sulfate concentration.

One sample of RA-glutenin (2.5 mg/ml) was fractionated with ammonium sulfate at a concentration of 200 mg/ml to obtain two distinct fractions in sufficient quantities for further characterization (Table II, $P_1$ and $S_1$). The precipitate fraction ($P_1$) contained subunits in the 80,000–134,000 mol wt region and 35,000 and 45,000 mol wt subunits (Fig. 5B). The supernatant fraction ($S_1$) contained subunits in the 68,000–12,000 mol wt region (Fig. 5H).

Fractions $P_1$ and $S_1$ were characterized further by gel filtration (Fig. 6) and amino acid composition analysis (Table III).

Gel filtration of $P_1$ on Sephadex G-200 with AU solvent (Fig. 6A) gave three peaks with I eluting with the void volume. Peak I contained approximately 12% of the protein applied to the Sephadex column (Table IV). SDS-PAGE showed that peak I (Fig. 5C) contained subunits predominantly in the 35,000–68,000 mol wt region, peak II (Fig. 5D) in the 68,000–134,000 mol wt region, and peak III (Fig. 5E) contained 35,000 and 45,000 mol wt subunits.

The elution profile for the $S_1$ (supernatant) fraction (Fig. 6B) was similar to that of $P_1$; however, there was substantially less protein in the peaks II and III fractions (see Table IV). Again, peak I eluted with the void volume.

SDS-PAGE showed that peak I of $S_1$ (Fig. 7D) contained essentially the same subunits as peak I of total RA-glutenin (Fig. 7B). The patterns of $S_1$ (Fig. 7C) and peak I of $S_1$ (Fig. 7D) were essentially the same. Those of peaks II and III of $S_1$ (Fig. 7E and F) were quite different, but there was considerable overlap.

$P_1$ was redissolved in AUC (2.5 mg/ml), precipitated once more with ammonium sulfate (200 mg/ml), and centrifuged to give a supernatant fraction, $S_2$, and a precipitate fraction, $P_2$. $S_2$ comprised approximately 20% and $P_2$ 76% of the protein of $P_1$.

SDS-PAGE showed that $P_2$ (Fig. 5F) contained subunits with mobilities similar to those of $P_1$ (Fig. 5B). On the other hand, $S_2$ (Fig. 5G) had a slightly different pattern than that of $S_1$ (Fig. 5H). $S_2$ contained more intensely stained subunits in the 68,000–90,000 mol wt region.

The results from the ammonium sulfate fractionation experiments indicate that peak I subunits (mol wt 68,000–12,000)

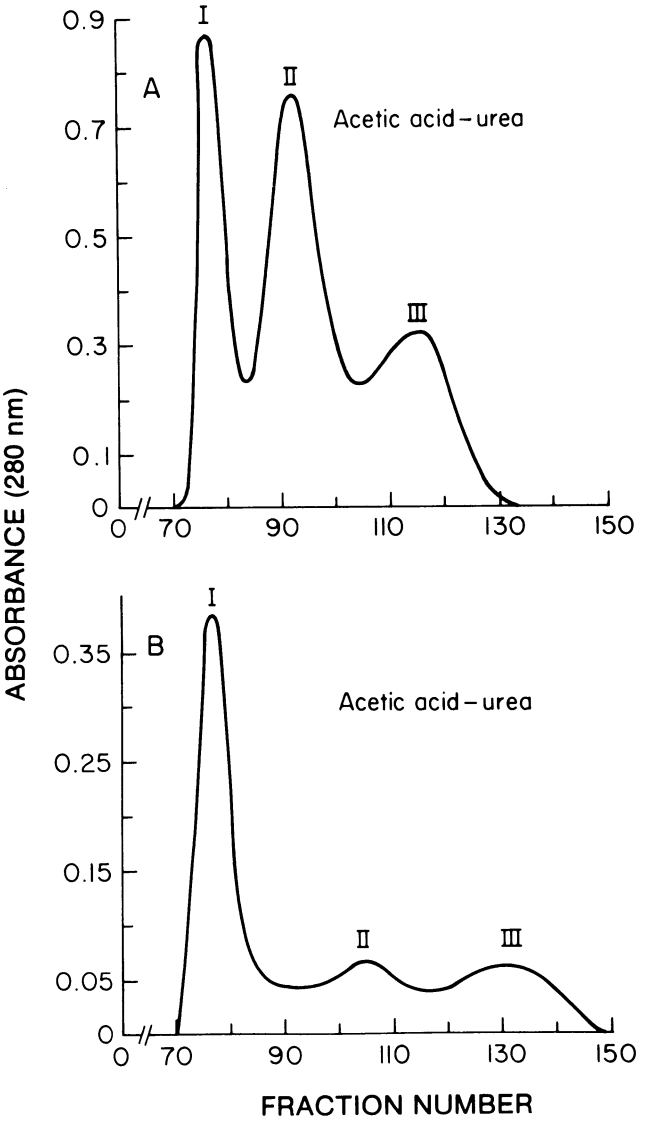


**Fig. 6.** Gel filtration on Sephadex G-200 in **A,** acetic acid-urea solvent of precipitate, $P_1$ and **B,** supernatant $S_1$ from fractionation with ammonium sulfate of RA-glutenin. Column, 5.0 × 100 cm; bed height, 72 cm; sample concentration, 12.5 mg/ml; flow rate, 45 ml/hr.

**TABLE III**

**Amino Acid Composition (mol %) of Fractions from Gel Filtration of Precipitate ($P_1$) and Supernatant ($S_1$) Isolated from Ammonium Sulfate Fractionation of RA-Glutenin (cv. Manitou)**

| | $P_1$ | | | | $S_1$ | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Amino Acid[a] | Total | I | II | III | Total | I | II | III | RA-Glutenin |
| Lysine | 1.1 | 3.6 | 0.8 | 0.6 | 5.6 | 5.2 | 5.6 | 5.7 | 1.9 |
| Histidine | 1.5 | 2.1 | 0.9 | 1.5 | 2.4 | 2.3 | 2.6 | 2.3 | 1.7 |
| Arginine | 3.5 | 4.7 | 2.3 | 3.8 | 6.8 | 5.9 | 7.1 | 6.0 | 4.0 |
| Aspartic acid | 2.4 | 7.5 | 1.0 | 1.5 | 9.2 | 9.3 | 8.0 | 9.7 | 3.5 |
| Threonine | 2.8 | 3.9 | 2.9 | 2.3 | 4.7 | 4.5 | 4.4 | 3.9 | 3.2 |
| Serine | 6.3 | 6.4 | 6.2 | 6.4 | 6.6 | 7.0 | 6.6 | 6.8 | 6.3 |
| Glutamic acid | 37.3 | 20.4 | 39.4 | 40.4 | 15.9 | 14.2 | 16.8 | 14.6 | 32.2 |
| Proline | 13.6 | 8.3 | 12.7 | 15.3 | 5.4 | 6.1 | 5.9 | 5.6 | 12.2 |
| Glycine | 7.8 | 8.9 | 16.0 | 3.2 | 8.9 | 8.8 | 8.9 | 8.7 | 9.0 |
| Alanine | 3.0 | 6.8 | 2.9 | 2.2 | 7.8 | 8.6 | 8.2 | 8.8 | 4.1 |
| Valine | 3.7 | 5.9 | 2.1 | 4.1 | 5.9 | 6.6 | 6.0 | 6.8 | 4.1 |
| Methionine | 1.0 | 2.1 | 0.6 | 1.7 | 1.7 | 1.9 | 1.6 | 1.7 | 1.6 |
| Isoleucine | 2.9 | 3.9 | 1.3 | 3.6 | 4.3 | 4.4 | 4.0 | 4.5 | 3.0 |
| Leucine | 6.5 | 8.5 | 4.8 | 7.2 | 8.2 | 8.6 | 7.5 | 8.3 | 6.8 |
| Tyrosine | 2.7 | 3.3 | 4.6 | 1.4 | 2.5 | 2.6 | 2.6 | 2.8 | 3.0 |
| Phenylalanine | 3.5 | 3.7 | 1.2 | 4.8 | 3.7 | 3.6 | 3.8 | 3.4 | 3.4 |

[a]Cysteine and tryptophan were not determined.

aggregate whenever the concentration of these subunits increases to a certain minimum level. If this occurs in any experimental operation such as freeze-drying or sample preparation for gel-filtration chromatography, the subunits aggregate. Gel filtration results on the original RA-glutenin and $S_1$ and $S_2$ fractions all support this hypothesis.

**Carbohydrate Content**

To investigate the possibility that aggregation of peak I subunits may involve carbohydrates, the carbohydrate content of each peak from gel filtration was determined. Andrews (1965) showed that certain glycoproteins, eg, human $\gamma$-globulins, exhibit abnormal behavior in gel-filtration chromatography. That study showed that proteins that contained as little as 2% carbohydrate gave apparent molecular weight values (by gel filtration) that were about 30% higher than the values obtained by other methods. Furthermore, Danno et al (1978) observed that the ethanol-insoluble fraction I of glutenin (from pH precipitation of reduced cyanoethylated glutenin), which contained subunits with similar mobilities on SDS-PAGE as the peak I fraction from gel filtration, contained 30% carbohydrate.

Of the three gel-filtration fractions of RA-glutenin, peak I had the highest carbohydrate content, 6.5%; peaks II and III contained 1 and 0.3%, respectively. The original RA-glutenin contained 4.9% carbohydrate. Accordingly, carbohydrates apparently may be involved in aggregation of specific subunits to form the peak I fraction. The nature of the carbohydrate-protein interactions remains to be discovered.

**Isoelectric Focusing**

Isoelectric focusing was selected as the technique in an attempt to isolate individual subunits of glutenin for further characterization. This technique was first used by Mita and Yonezawa (1971) to isolate subunits from total RA-glutenin. In the present study, the technique was applied to peak II fraction. This fraction was selected because it contains subunits that appear to be important in bread-making quality of common wheats (Orth and Bushuk 1973c). Furthermore, the subunits of this fraction appear to be unique because of their high molecular weight (by SDS-PAGE) and peculiar amino acid composition (Table V).

Preliminary experiments with 3–10 and 5–9 pH gradients of carrier ampholytes were unsuccessful in isolating individual subunits. These experiments showed that all of the peak II subunits focused in the 6–8 pH range. Accordingly, the peak II fraction was then focused on a narrower pH gradient, 6–8 (6*M* urea for five days at 600 V). The UV absorption profile (Fig. 8) showed

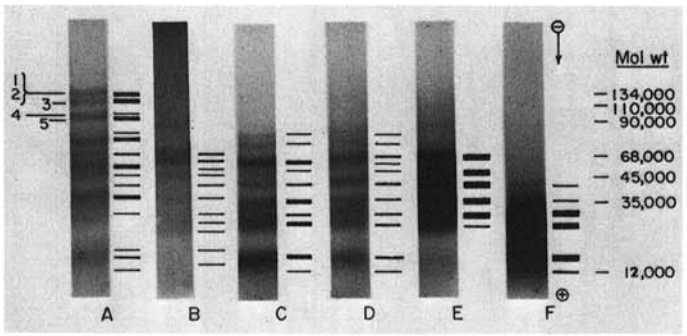


**Fig. 7.** SDS-PAGE at pH 8.4 of $S_1$ (supernatant from ammonium sulfate fractionation) and fractions from gel filtration of $S_1$: **A,** RA-glutenin; **B,** peak I from gel filtration of RA-glutenin; **C,** $S_1$; **D,** peak I from gel filtration of $S_1$; **E,** peak II from $S_1$; and **F,** peak III from $S_1$. (**1–5** on the left refer to the first five high molecular weight subunits of glutenin.)

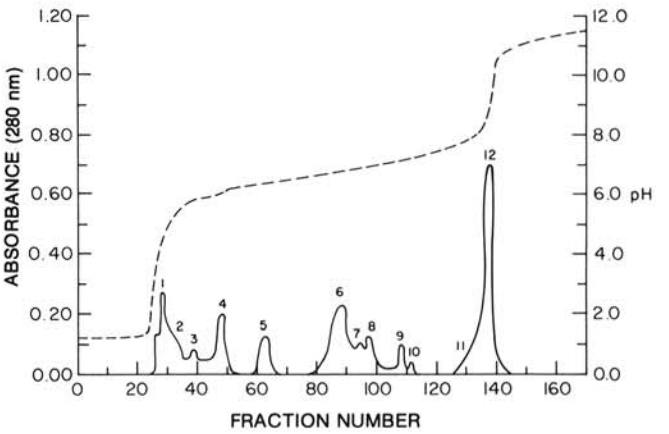


**Fig. 8.** Elution profile of peak II fraction (from gel filtration of RA-glutenin) separated by isoelectric focusing at pH 6–8. Solvent, 6*M* urea; time, 120 hr; voltage, 600 V; sample, 40 mg. - - - = pH, — = absorbance.

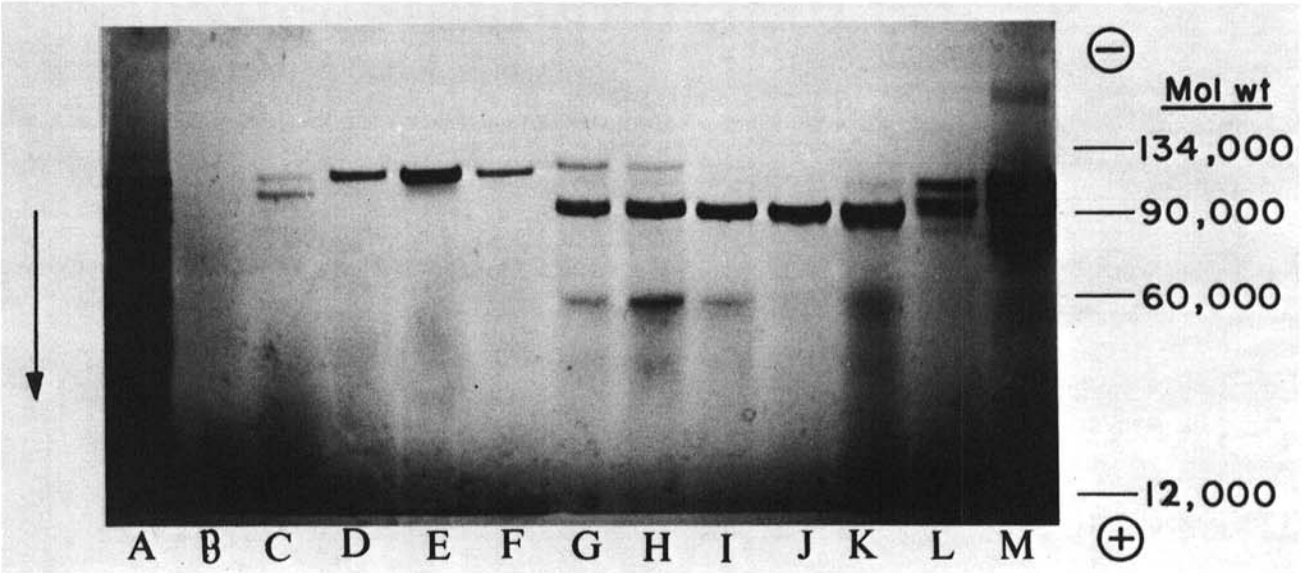


**Fig. 9.** SDS-PAGE at pH 8.4 of fractions from isoelectric focusing of peak II fraction from gel filtration: **A,** RA-glutenin; and **B–M,** fractions 1 to 12 of Fig. 8, respectively.

approximately 12 peaks in the range of pH 5–9. SDS-PAGE (Fig. 9) of each peak showed that fractions with distinctly different pIs contained many subunits with the same apparent molecular weight (68,000–134,000). This indicates that glutenin contains subunits of similar molecular weight but different amino acid composition. The 12 fractions contained approximately 20 different protein species by SDS-PAGE. Danno et al (1976) also demonstrated charge heterogeneity by isoelectric focusing on polyacrylamide gels of subunits of glutenin that appeared homogeneous by SDS-PAGE. Two fractions from electrofocusing, peak 3 (Fig. 9D) and peak 9 (Fig. 9J), appeared to be homogeneous, containing subunits of 134,000 and 90,000 mol wt, respectively. These were therefore isolated for further characterization.

### N-Terminal Amino Acid Analysis

The homogeneity of the two subunits isolated by isoelectric focusing (peaks 3 and 9, Fig. 8, or pattern D and J, Fig. 9) was checked by N-terminal amino acid analysis according to the method of Zanetta et al (1970). The N-terminal amino acids of the 134,000 and 90,000 mol wt subunits were aspartic acid and glutamic acid, respectively. Huebner et al (1974) reported that the N-terminal amino acid for the highest molecular weight (133,000) subunit of glutenin (their glutenin fraction B-1 of common wheat cultivar Ponca) was glutamic acid, whereas Mita and Yonezawa (1971) found that the N-terminal amino acid for their highest molecular weight subunit (their glutenin fraction P of common wheat cultivar Norin No. 26) was alanine. The differences among the three studies could be due to intercultivar variability (genotypic) or to the fact that the subunits isolated may be different (methods of preparation were different).

### Amino Acid Composition

Amino acid compositions of the fractions from gel filtration in AU solvent of RA-glutenin (Table V) are very similar to those obtained previously by Hamauzu et al (1975) and Huebner et al (1974). Peak I proteins contain higher proportions of aspartic acid and basic and hydrophobic amino acids than do peak II and III proteins. In this respect, peak I proteins resemble wheat albumins and globulins (Huebner et al 1974).

Peak II proteins have relatively low contents of basic, acidic, and hydrophobic amino acids but high contents of glutamic acid, glycine, and proline. On the other hand, peak III proteins resemble gliadin proteins in amino acid composition (Huebner et al 1974) in having high contents of glutamic acid and proline and a low glycine content.

Table III shows the amino acid composition of fractions $P_1$ and $S_1$ from ammonium sulfate fractionation and also the fractions obtained from gel filtration of $P_1$ and $S_1$. Differences in amino acid composition are evident between $P_1$, $S_1$, and RA-glutenin, especially in the basic amino acids and in aspartic and glutamic acids. $P_1$ and $S_1$ show marked differences in amino acid composition. The three peaks obtained from gel filtration of $P_1$ have amino acid compositions similar to the three peaks obtained from gel filtration of RA-glutenin (Tables III and V). On the other hand, $S_1$ has higher contents of basic amino acids, aspartic acid, and hydrophobic amino acids than does $P_1$, RA-glutenin, and the three peaks obtained from gel filtration of $P_1$ and RA-glutenin. The three peaks obtained from gel filtration of $S_1$ (Table III) have similar amino acid compositions to that of $S_1$.

Table VI shows the amino acid composition of the fractions obtained by isoelectric focusing. Significant differences were noted among fractions in the content of the acidic and basic amino acids. Variations were wider in the content of glutamic acid, proline, and glycine, which fluctuated among fractions from 34 to 40, 12 to 19,

TABLE IV
**Quantitation of Protein Fractions from Gel Filtration on Sephadex G-200 of Precipitate ($P_1$) and Supernatant ($S_1$) (From Ammonium Sulfate Fractionation of RA-Glutenin)**

| Fractions | Supernatant ($S_1$) (%) | Precipitate ($P_1$) (%) |
|---|---|---|
| Peak I | 70 | 12 |
| Peak II | 12 | 42 |
| Peak III | 15 | 44 |

TABLE V
**Amino Acid Composition (mol%) of RA-Glutenin and Three Fractions Obtained from it by Gel Filtration on Sephadex G-200**

| | RA-Glutenin | | | |
|---|---|---|---|---|
| Amino Acid[a] | Total | I | II | III |
| Lysine | 1.9 | 3.8 | 0.7 | 0.8 |
| Histidine | 1.7 | 2.0 | 0.7 | 1.6 |
| Arginine | 4.0 | 4.8 | 2.1 | 3.7 |
| Aspartic acid | 3.5 | 7.6 | 0.7 | 2.3 |
| Threonine | 3.2 | 4.1 | 3.0 | 2.7 |
| Serine | 6.3 | 6.5 | 6.3 | 6.5 |
| Glutamic acid | 32.2 | 20.0 | 40.4 | 39.3 |
| Proline | 12.2 | 7.9 | 12.3 | 15.0 |
| Glycin | 9.0 | 9.8 | 17.7 | 3.6 |
| Alanine | 4.1 | 7.0 | 2.8 | 2.7 |
| Valine | 4.1 | 5.8 | 1.6 | 4.3 |
| Methionine | 1.6 | 1.8 | 0.3 | 0.7 |
| Isoleucine | 3.0 | 3.9 | 0.9 | 3.7 |
| Leucine | 6.8 | 8.3 | 4.3 | 7.3 |
| Tyrosine | 3.0 | 3.4 | 5.4 | 1.4 |
| Phenylalanine | 3.4 | 3.4 | 0.6 | 4.4 |

[a]Cysteine and tryptophan were not determined.

TABLE VI
**Amino Acid Composition (mol %) of Fractions from Electrofocusing of Peak II Fraction (Gel Filtration) of RA-Glutenin**

| Amino Acid[a] | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 12 |
|---|---|---|---|---|---|---|---|---|---|
| Lysine | 1.4 | 1.1 | 2.0 | 1.9 | 1.6 | 1.3 | 1.4 | 1.8 | 0.4 |
| Histidine | Trace | 0.4 | 0.7 | 0.9 | 1.2 | 1.1 | 1.3 | 1.3 | 0.8 |
| Arginine | Trace | 0.9 | 0.7 | 0.7 | 1.8 | 1.5 | 2.0 | 1.0 | 1.6 |
| Aspartic acid | 1.5 | 1.0 | 1.4 | 2.7 | 2.6 | 3.1 | 1.9 | 1.4 | 1.0 |
| Threonine | 2.8 | 3.0 | 3.0 | 3.3 | 3.0 | 3.1 | 3.2 | 3.4 | 3.2 |
| Serine | 7.4 | 6.5 | 7.2 | 8.4 | 6.9 | 6.8 | 7.5 | 7.2 | 7.3 |
| Glutamic acid | 38.8 | 40.6 | 38.6 | 36.5 | 34.6 | 38.0 | 37.4 | 36.6 | 38.4 |
| Proline | 19.9 | 14.1 | 12.5 | 13.8 | 14.6 | 14.3 | 13.8 | 15.6 | 13.0 |
| Glycine | 18.1 | 19.3 | 20.4 | 15.2 | 11.4 | 11.1 | 12.8 | 16.6 | 19.0 |
| Alanine | 2.2 | 2.5 | 2.8 | 3.2 | 3.1 | 2.8 | 2.9 | 2.9 | 2.9 |
| Valine | Trace | 1.1 | 1.1 | 2.5 | 3.2 | 3.3 | 3.0 | 2.2 | 1.6 |
| Methionine | Trace | Trace | Trace | Trace | Trace | Trace | 0.3 | Trace | Trace |
| Isoleucine | 0.8 | 0.7 | 1.2 | 2.1 | 3.6 | 2.7 | 2.1 | 1.3 | 1.0 |
| Leucine | 3.8 | 4.1 | 4.5 | 5.2 | 7.4 | 5.4 | 5.8 | 4.4 | 3.6 |
| Tyrosine | 3.5 | 4.6 | 4.1 | 2.7 | 4.0 | 2.7 | 3.2 | 3.7 | 5.7 |
| Phenylalanine | Trace | 0.1 | Trace | 1.0 | 2.0 | 1.9 | 1.3 | 0.6 | 0.2 |

[a]Cysteine and tryptophan were not determined.

and 11 to 20 mol %, respectively. Similar variations in amino acid composition were obtained by Huebner et al (1974) for three fractions of the high molecular weight subunits of RA-glutenin of the cultivar Ponca. The two fractions (Fig. 8, peaks 3 and 9) that gave single bands by SDS-PAGE differ substantially in the proportions of arginine, proline, glycine, and valine.

## DISCUSSION

Gel filtration, electrofocusing, amino acid composition, salt fractionation, and SDS-PAGE results indicate that glutenin is considerably more complex than previously appreciated. The three fractions from gel filtraton differed markedly in physical (tendency to aggregate) and chemical (amino acid composition) properties. These differences may be relevant to functional properties.

Peak I fraction from gel filtration contains subunits that have a strong tendency to aggregate. This peculiar behavior may result from the unique amino acid composition, eg, high contents of basic, acidic, and hydrophobic amino acids, or from the presence of a relatively high amount of carbohydrate. The implication of carbohydrates in glutenin aggregation warrants further investigation. The fact that solvent containing SDS disaggregated peak I proteins to a much larger extent than the other solvents investigated in this study suggests that hydrophobic interactions play an important role in the aggregation behavior of these proteins.

Peak II subunits are unique both in size and chemical composition; they have molecular weights of 68,000–134,000 and contain relatively high proportions of glycine, an amino acid that is predominant in structural proteins such as collagen. Peak III subunits, on the other hand, resemble gliadin proteins in molecular weight (35,000 and 45,000) and amino acid composition.

It is postulated that peak II subunits play a central role in dough structure by forming a covalent network, through disulfide bonding, in which the subunits of peaks I and III are held through secondary forces to form the glutenin complex. This hypothesis is consistent with the model of functional glutenin proposed previously (Khan and Bushuk 1979).

The ammonium sulfate fractionation studies revealed that the aggregation behavior of peak I subunits depends strongly on concentration. It is important to keep this in mind whenever operations such as freeze-drying and gel filtration are used in research on glutenin proteins.

Isoelectric focusing of peak II proteins revealed a further complexity of glutenin. Use of a narrow pH gradient gave a better resolution and separation of peak II subunits than previously achieved by ion-exchange chromatography (Huebner and Wall 1974) or electrofocusing of cyanoethylated glutenin on a 3–10 pH gradient (Mita and Yonezawa 1971). The fact that many subunits of the same molecular weight (by SDS-PAGE) can be separated by electrofocusing indicates that native (unreduced) glutenin is highly heterogeneous in amino acid composition. These findings are consistent with those of Bushuk and Wrigley (1971), Huebner and Wall (1976), and Orth et al (1973), who provided some evidence on the polydispersity of glutenin from their gel-filtration elution profiles of unreduced glutenin. In the present study, two of the largest subunits of glutenin of the hard red spring wheat Manitou were isolated by isoelectric focusing and their N-terminal amino acids and amino acid compositions determined.

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