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Evidence for Glutenin in Wheat: Stability Toward Dissociating Forces[1]

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ABSTRACT

The hypothesis that glutenin is an artifact of classic isolation procedures was examined by extraction of wheat proteins into urea solutions or phenol-acetic acid-water mixtures and by gel filtration of glutenin after sonication. After extraction of flour with water and then 2M urea, nitrogen recovered in dialyzed extracts and in the starch residue totaled 127% of that originally in the flour; 43% remained in the residue. Lyophilization and redialysis of all urea extracts and the residue lowered nitrogen recovery to 98% with 34% remaining in the residue. Nitrogen in the residue was reduced to 17% of the original amount by further extraction with 0.2N acetic acid. Subsequent treatment of the residue with mercaptoethanol and acrylonitrile and extraction with dilute acetic acid left less than 6% of the nitrogen insoluble. Gel filtration and gel electrophoresis showed that all urea extracts contained glutenin-like material along with low-molecular-weight proteins. Final extractions with dilute acetic acid removed glutenin with only a trace of gliadin. Ultrasonics dissociated glutenin and made it tractable enough to be separated on agarose columns in urea buffer at pH 9.2. The results support the contention that glutenin is highly aggregated and different from gliadin. The data show that urea extraction does not remove all protein from wheat flour.

Since the work of Osborne (1), the high-molecular-weight (MW) fraction of wheat gluten, which is insoluble in 70% ethanol, has been identified by the term glutenin. Since then, different extraction methods have been devised that give varying percentages of gliadins, glutenins, and albumins without a clear-cut distinction between the three groups. These proteins are perhaps best separated on the basis of differences in their solubilities and gel-filtration properties (2,3).

The inability of most methods to extract all the protein from wheat flour has also prompted exploration of new procedures that could remove the protein without changing it irreversibly. The tendency of classic glutenin to form insoluble aggregates, coupled with the belief that glutenin might be an artifact of the extraction procedure, led Lee (4) to extract wheat protein with 2M urea. Lee's

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conclusion that 2M urea removes all gluten proteins—mainly as gliadin—contrasts sharply with the concept that wheat gluten contains nearly equal proportions of two different types of proteins (3). Seeking a better understanding of this dilemma, we have further studied the preparation of wheat proteins by extraction and have explored the effect of sonication upon high-MW protein components from wheat flour.

MATERIALS AND METHODS

All chemicals were ACS grade and were used as received. Urea was purchased from the Mallinckrodt Chemical Works.

Forty grams of defatted (with dry butanol) Ponca hard red winter wheat flour was extracted twice with 150-ml. volumes of deionized water and six times with 150-ml. volumes of 2M urea. Extractions with urea solutions were performed according to the method of Lee (4). After 15 min. of centrifugation at 2,400 X g (ca. 5°C.), each supernatant was removed and dialyzed individually against 4 liters of distilled water for 3 days at room temperature with six to seven changes of water, after which each supernatant was lyophilized separately.

Detection of urea in dialysis water was essentially as described by Feigel (5). Dialysis water was first concentrated to about one-fifth volume and then two drops of 5% phenolphthalein (in EtOH) solution were added to 5 ml. of the water. The solution was titrated with 0.01N NaOH to a slightly pink color and then carefully back-titrated with 0.001N HCl until it became colorless. Two drops of 5% urease in water were added to this solution, which turned pink in 5 to 10 min. from the release of ammonia if urea were present. This method gave a positive pink color with less than 0.05 mg. urea per ml. (8×10^{-4}M).

Gluten was also extracted with phenol:acetic acid:water (PAW) (1:1:1) according to Gallus and Jennings (6). After dialysis to eliminate phenol, gliadin and glutenin were prepared from the dried gluten by dissolving in 0.1N acetic acid and precipitating from 70% EtOH as by Jones et al. (7).

Nitrogen was determined by micro-Kjeldahl.

Electrophoresis was in starch gel with 3M urea, aluminum lactate buffer at pH 3.1, according to Huebner et al. (8).

Sephadex G-100 (Pharmacia Fine Chemicals, Inc.), Bio-Gel P-300, Bio-Gel A-0.5m, A-1.5m, and A-5m (Bio-Rad Laboratories) were used in gel filtration.

Low-power sonication of glutenin was carried out with an ultrasonic laboratory glassware cleaner. Higher power sonication was achieved with a Branson sonifier, Model 125, equipped with a 0.5-in. diameter probe. Glutenin samples of 40 to 50 mg. suspended in 4 to 5 ml. of buffer in a 30-ml. beaker were sonicated for 10 to 15 min. at room temperature in the ultrasonic cleaner. The beaker containing the sample was positioned near the center of the tank at a depth that gave maximum visible agitation of the solution. When the Branson sonifier was operated at 50-w. power at a frequency of 20 kHz, a small sample turned almost instantly to a thick foam, which could be partially controlled with a drop of octyl alcohol. Temperature increase produced with the Branson sonifier was not sufficient to require external cooling during the 1 to 2 min. needed to solubilize the sample. Before chromatography, the solutions were centrifuged in a clinical centrifuge to remove any undissolved material and to prevent plugging of the column.

For amino acid analyses of the starch residue, 200 mg. of sample was refluxed in

200 ml. constantly boiling HCl for 24 hr. An appropriate sample was analyzed by an automatic amino acid analyzer as previously described (9).

RESULTS

Urea Extraction

Table I gives the nitrogen recovered in water and urea extracts. Even though the fractions were dialyzed for 3 days, nitrogen recoveries totaled 127% of that in the starting material, with 43% remaining in the starch residue. Also, some of the last extracts (e.g., urea 4, 5, and 6) contained more than 20% nitrogen. Since pure gliadin contains only 17.5% nitrogen, undialyzed urea or carbamalytion was suspected. The samples, previously lyophilized, were resuspended in water and redialyzed for a day with several changes of water to determine whether the additional nitrogen was firmly bound. After redialysis and lyophilization, nitrogen recoveries (Table I) totaled 98%.

TABLE I. PERCENT NITROGEN EXTRACTED FROM WHEAT FLOUR[a]

	%N	
Extractant	Dialysis 3 days	Additional dialysis
Water		
1	10	
2	6	
2M Urea		
1	37	36
2	10	6
3	6	>2
4	5	<2
5	5	>1
6	5	<1
N left in starch	43	34
Total N recovered	127	98

[a]Owing to the many manipulations of the sample, confidence limits of values given for the last four urea extractions are ±10 to 15%.

Extraction experiments on smaller samples under conditions designed to facilitate faster dialysis gave essentially the same results. After 4 days of dialysis, the last dialysis water from overnight showed no urea present. Less excess nitrogen was found with the samples, but the final urea extracts and the starch residue contained additional nitrogen that was removed only after lyophilization and redialysis.

In Fig. 1 the nitrogen recovered in each extraction before and after additional dialysis is compared to recoveries by Lee (4). Our data resemble those of Lee in that nitrogen recoveries after one 3-day series of dialyses remained near 5% in the later extracts. After the extracts were dried and redialyzed, however, nitrogen levels diminished and approached 0% in the later extracts.

Part of the 34% nitrogen that remained in the starch residue apparently was protein. Amino acid composition of this protein on a molar ratio basis fell between

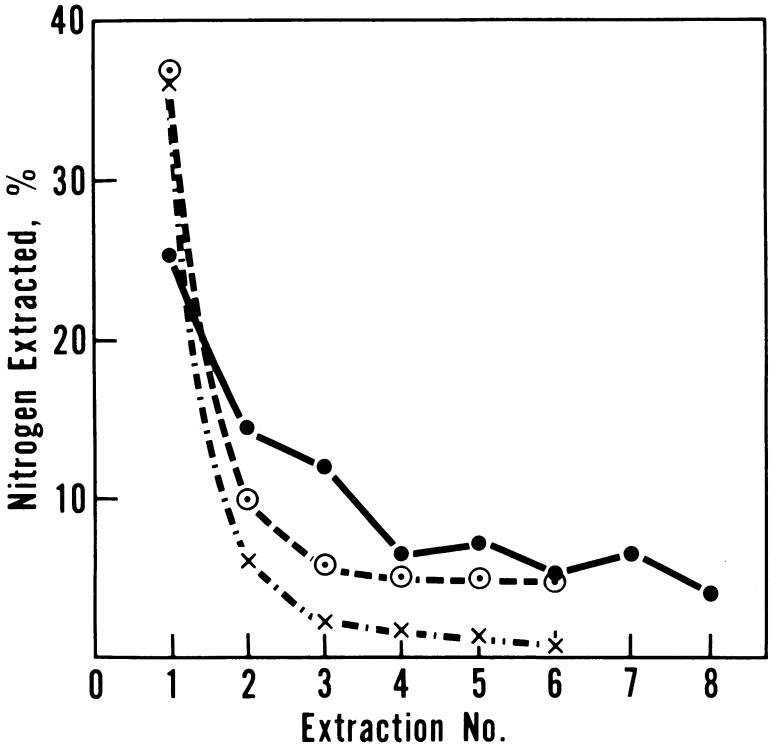

Fig. 1. Comparisons of percentage of nitrogen extracted from wheat flour with 2M urea. Open circles, after 3 days' dialysis; letter X, after lyophilization, resolution, and dialysis for one additional day; closed circles, percentage of protein nitrogen extracted by Lee (4).

that of glutenin and that of the acetic acid-insoluble fraction described by Cluskey and Dimler (10). Extraction of a portion of the starch residue with 0.2N acetic acid removed 12 to 14% of the original nitrogen and left 16 to 17% in the residue. The remaining residue was treated with 0.1 ml. mercaptoethanol for 30 min. in 5M urea at pH 8.0; then for 15 min. with 0.5 ml. acrylonitrile; and finally extracted with 0.2N acetic acid. This treatment removed another 11 to 13% nitrogen and left less than 6% of the original nitrogen in the residue.

Gel-filtration properties of some of the extracts are shown in Fig. 2. We found that Sephadex G-100 gives a better separation of the gluten fractions than does Bio-Gel P-150, although recent modification of the Bio-Gels by the manufacturer may give cause to reconsider this observation. Slightly longer columns than Lee used allowed larger samples and reduced the percentage error due to loss of a few milligrams or inaccuracies with low concentrations. Also, 0.2N acetic acid was used with Sephadex filtration for convenience and economy, and because it promoted uniformity in lyophilized samples and gave sharper peaks and better resolution than did the use of acetate buffers (pH 4 to 5) either with or without urea. All extracts, including the second water extract, showed some glutenin. The ratio of glutenin to gliadin generally increased with each extract, whereas the gluten protein content of

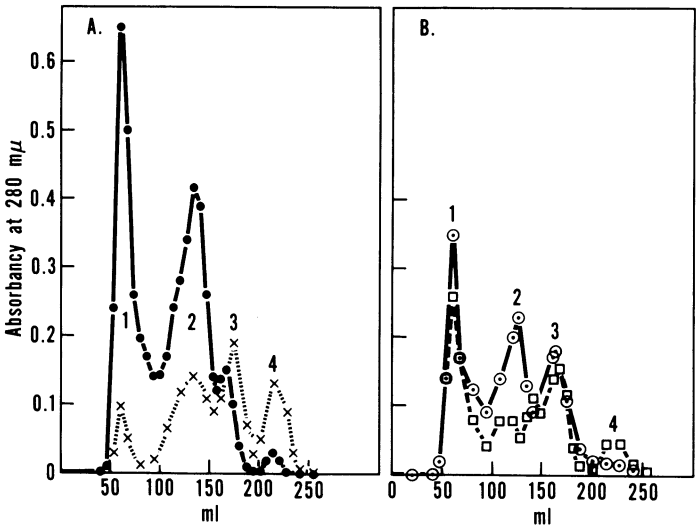

Fig. 2. Gel filtration (with Sephadex G-100) of various extracts of wheat flour. Column, 2.2 X 58 cm.; buffer, 0.2N acetic acid; sample size, 60 mg. Peaks are, in order: 1) glutenin, 2) gliadin, 3) albumin, and 4) other nonprotein material. A. Letter X, water extraction No. 2; closed circles, 2M urea extraction No. 1. B; Open circles, 2M urea extraction No. 3; squares, 2M urea extraction No. 5.

the material extracted decreased, as is evident by the smaller glutenin and gliadin peaks from urea extracts 3 and 5 in Fig. 2, B. One exception is the albumin peak at about 175 ml. (Fig. 2, B) from urea extract number 5, which divided into two and did not decrease in size like the gliadin and glutenin peaks.

Figure 3 shows that the acetic-acid extract contains glutenin predominantly, with only a trace of gliadin and albumin. Gel filtration of the acetic-acid extract, after reduction and alkylation of the starch residue, was similar to Fig. 3. Electrophoretic patterns on starch gels confirmed the identities of the gel-filtration fractions and disclosed that most of the protein extracted from the starch residue with acetic acid did not enter the gel until after reduction, when it gave the gel pattern characteristic of reduced glutenin.

Phenol Extraction

Extraction of wheat flour with PAW (1:1:1) removed 97 ± 2% nitrogen. The glutenin fraction was subjected to gel filtration on Sephadex G-100 and Bio-Gel P-300 (column 2.2 X 50 cm.) using PAW (1:1:2). PAW containing less water did not expand the gel sufficiently to allow reasonable separation in the MW range fractionated by those gels in aqueous solutions. Since almost all the glutenin was eluted in a single peak at the void volume, the MW may be more than 100 to 200,000. Starch-gel electrophoresis of reduced and alkylated glutenin prepared by PAW extraction appeared to be the same as glutenin prepared by other methods. Gliadin subjected to filtration on Sephadex G-100 and chromatography on sulfoethyl cellulose also gave results similar to gliadin prepared by previous methods.

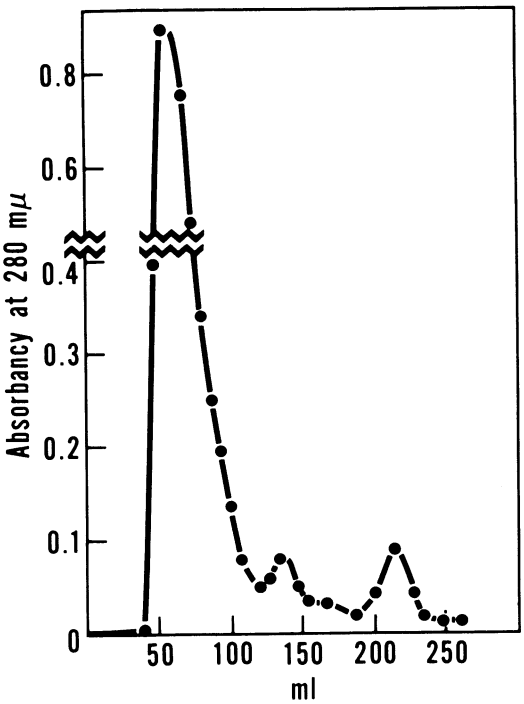

Fig. 3. Gel filtration of 0.2N acetic acid extract of starch residue after previous extractions with 0.2M urea. Column, 2.2 × 58 cm. Sephadex G-100; buffer, 0.2N acetic acid; sample size, 70 mg.

Sonication

The insoluble nature of isolated glutenin and the high viscosity of glutenin dispersions in dissociating solvents make gel filtration of this protein extremely difficult. Crow and Rothfus (11) were able to chromatograph glutenin in 8M urea on a gel stabilized by inert support but at rather slow flow rates. Glutenin subjected to ultrasonic oscillation became much more soluble and could be passed through filtration columns quite readily.

The same gel-filtration elution pattern was obtained with glutenin regardless of which instrument was used to disperse the sample. Various filtration columns were tried including Sephadex G-100 and G-200, Bio-Gel P-300, and the agarose gels, Bio-Gel A-0.5m to A-5m. Generally glutenin emerged unretarded and unresolved. Whereas the protein could be chromatographed in acidic buffers with the Sephadex and Bio-Gel P-300 columns, it adhered to the agarose gels until the pH was raised to above 8.0 or until 0.1M NaCl was added to the buffer, similar to the experience of Inamine et al. (12). Unfortunately, the partial solubility of agarose gels in high concentrations of urea and guanidine hydrochloride prevented the use of these reagents. A buffer that fit all requirements best was 2 or 3M urea–tris(hydroxymethyl)aminomethane (THAM). Gel filtration in 3 or 5M guanidine hydrochloride buffers did not improve the separation over that achieved with 3M urea.

Figure 4 contains some results obtained with agarose columns. With Bio-Gel A-0.5m and A-1.5m (Fig. 4, A and B), there was little separation except for a

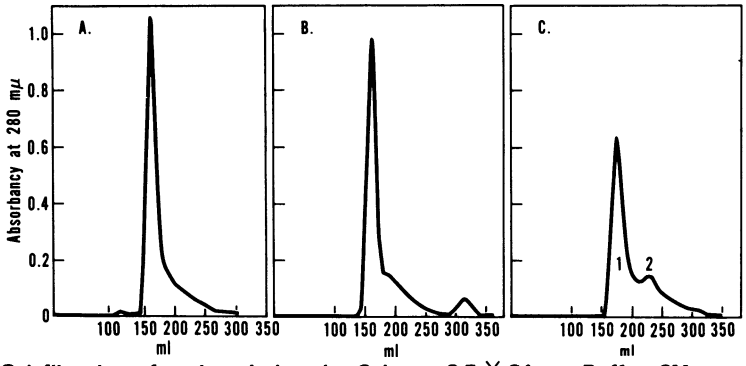

Fig. 4. Gel filtration of sonicated glutenin. Column, 2.5 × 84 cm. Buffer: 3M urea, 0.016M tris(hydroxymethyl)aminomethane (THAM), and 0.002M HCl, pH 9.2. A. Bio-Gel A-0.5m; B; Bio-Gel A-1.5m; and C. Bio-Gel A-5m.

shoulder following the main peak. This shoulder contained a lower-MW glutenin and a trace of gliadin, which migrated into a starch gel readily during electrophoresis. Most of the glutenin from the main peak did not enter the starch gel. On an A-5m gel, which has a 5 million exclusion limit (Fig. 4, C), there was a definite separation of the two fractions. After reduction and alkylation, these two fractions differed in that one of the slower-moving bands commonly seen in electrophoretic patterns of reduced and alkylated glutenin was missing from the higher-MW fraction (Fig. 5). There were also slight quantitative differences of the other components between the two fractions.

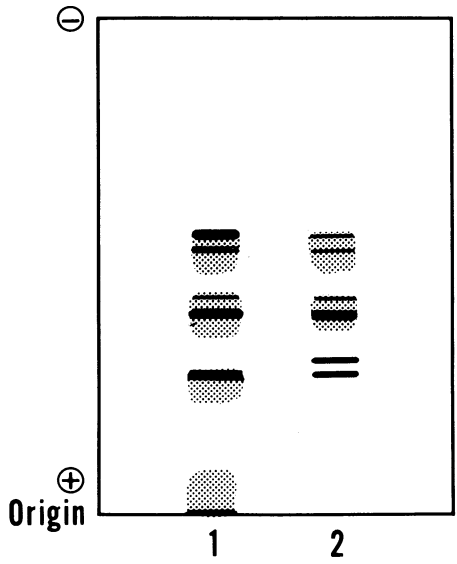

Fig. 5. Starch-gel electrophoresis of reduced and alkylated fractions from Fig. 4, C.

DISCUSSION

Nearly all quantitative protein determinations suffer from certain shortcomings that can affect the interpretation of protein-extraction data. In our case, where protein concentrations were calculated from Kjeldahl-nitrogen data, the presence of undialyzed urea or cyanates led to high recovery values. Had our nitrogen recoveries by chance been nearer 100%, error in the method would have been less obvious and we could have erroneously concluded that urea is an efficient extractant for gluten proteins. It is conceivable that deficiencies in other methods for protein analyses could cause similar difficulties. Indeed, with the Lowry method (13), which was used by Lee (4), the amount of color varies with different proteins, and is not directly proportional to concentration. Furthermore, Peters and Fouts (14) found that sucrose-containing samples, various buffers, and trace amounts of foreign materials give additional color with the Lowry method. We found that the last extracts with urea (e.g., 4, 5, and 6; Table I) were low in protein (less than 50%) and high in carbohydrate and urea. Perhaps Lee's results for the last urea extractions were also high. High nitrogen levels in our extracts could have been caused by carbamalytion (8,15). We have no evidence, however, that chemical substitution interfered with fractionation in our work.

We found that after all the water and urea extractions a large proportion of the more insoluble high-MW glutenin remains with the starch residue. No mention was made by Lee whether the starch residue was analyzed for remaining protein. This protein can be removed with dilute acetic acid, part before and part of it after reduction and alkylation. Our results did confirm Lee's observation that less high-MW material is extracted with 2M urea than with acetic acid; consequently, less material stayed at the origin upon starch-gel electrophoresis.

Extraction of wheat proteins by PAW according to Gallus and Jennings (6) was tried to determine whether the apparent low pH of 1.4 would have an adverse effect on either the gliadin or glutenin fractions. It was suspected that this low pH would cause some deamidation and possibly break peptide bonds. From our results on gel electrophoresis, gel filtration, chromatography, and reduction there was no noticeable change.

As yet, there is little information available on the sonication of cereal proteins. Godon and Petit (16), using sonication at various frequencies from 23 to 970 kHz, found somewhat variable quantitative differences among the fractions eluted from a Sephadex G-100 column. They suggest depolymerization of the glutenin fraction. Gallus and Jennings (6) used sonication in extracting protein from the wheat flour. Our purpose in sonication of the glutenin was to make it more soluble and more tractable for fractionation on gel-filtration columns.

The results (Fig. 4) suggest that one part of the sonicated glutenin (Peak 1) has a very high MW, possibly around 2 million (Blue Dextran is eluted at the same volume), and that the other part has a MW possibly around 1 million. The quantity of the two fractions depends on how the glutenin was prepared. Here the gliadin fraction had been removed by extracting twice with 70% ethanol, which also removed considerable quantities of the lower MW glutenin. Glutenin prepared by Sephadex filtration of gluten or extracted with 2M urea produces more equal quantities of the two fractions.

The exact reason for glutenin's becoming more soluble is not known. It could be

a simple deaggregation caused by extreme molecular agitation, since reduction and alkylation of glutenin, usually carried out in high concentrations of ureas for deaggregation, can also be performed without urea during sonication. Alternatively, sonic oscillation may impart sufficient energy to rupture certain covalent bonds. Considering variations in the behavior of different glutenin preparations after sonication, it seems likely that different bond energies are involved in the association of the glutenin proteins. Such a possibility might explain the French worker's observation of variable quantitative changes between gluten fractions after sonicating gluten at different frequencies.

Our work adds additional evidence to the work of others who have found that glutenin is chemically and physically different from gliadin and very likely is much higher in MW. The Maes (17) extraction procedure, which requires a weak acid or base to extract glutenin; the amino acid analysis by Wu and Dimler (18); gel electrophoresis, reduction, and alkylation by Woychik et al. (19); peptide analysis by Bietz and Rothfus (20); and gel filtration (2,3) have all shown that glutenin and gliadin differ.

Bakers have considerable difficulties with mixing and dough development because of different times required for various wheats. Since the glutenin fraction may be most responsible for these characteristics (21), it is important to develop methods for studying this group of proteins. With the new methods and tools that have become available the past few years, a renewed effort should soon give us some insight why physical and chemical characteristics differ among various varieties of wheat.

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