

Studies of Glutenin. I. Comparison of Preparative Methods¹

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ABSTRACT

Three preparative methods for glutenin, viz. Osborne fractionation, gel-filtration chromatography, and pH precipitation with subsequent purification using SE-Sephadex C-50 were evaluated. Amino acid analyses and disc-gel electrophoresis of the products showed that the third method yields glutenin that is free from low-molecular-weight contaminants. The dissociating solvent AUC (acetic acid, urea, CTAB) which dissolved 93% of the flour protein was used for the gel filtration and pH precipitation methods. Two other detergents, sodium dodecyl sulfate and Brij 35, in combination with acetic acid and urea, were less effective than CTAB and solubilized 60 and 89% of the flour protein respectively. Because of its relatively high solvent efficiency, and the fact that it is neutral, and so does not migrate on electrophoresis at pH 3.2, Brij 35 is preferable over CTAB as a protein dispersant for electrophoretic studies.

The relative insolubility of wheat glutenins has made their isolation and study extremely difficult. An increased awareness of their importance in breadmaking (1), and the fact that they represent up to 50% of the total flour protein, make the development of methods for their isolation and purification imperative. Introduction of the AUC solvent (water solution containing acetic acid-urea and hexadecyltrimethylammonium bromide CTAB) (2) was a major advance in this field as it can solubilize up to 98% of the total flour protein.

The aim of the present work was to determine the best method of obtaining pure glutenin (free of gliadin and other low-molecular-weight impurities) for subsequent molecular and structural characterization. Polyacrylamide gel electrophoresis and amino acid analyses were used as tests of purity.

MATERIALS AND METHODS

Flour Sample

The flour used was milled from a pure variety of hard red spring wheat (Manitou) on a Buhler experimental mill after overnight tempering to 15.5% moisture. It contained 12.9% protein (N X 5.7 on a 14.0% moisture basis), 0.40% ash, had a starch damage of 28.2 Farrand Units, and gave a loaf volume by the remix baking test of 860 cc. for 100 g. of flour.

Amino Acid Analysis

Protein samples (10 mg.) were weighed into hydrolysis tubes and hydrolyzed with 4 ml. of 6N hydrochloric acid under vacuum at 110°C. for 24 hr. The hydrolysate was dried over sodium hydroxide pellets in a vacuum desiccator. The residue was dissolved in 8 ml. of citrate buffer solution of pH 2.2 and centrifuged. A Beckman Model 121 amino acid analyzer was used for the

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analyses. Since most of the protein fractions analyzed contained nitrogenous compounds such as urea, the amino acid data were reported as mole percent on an ammonia-free basis.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was performed at pH 3.2 by the method of Davis (3) as modified by Chen and Bushuk (4). The gels were stained with Coomassie Brilliant Blue according to the directions of Koenig et al. (5).

RESULTS AND DISCUSSION

Modified Osborne Fractionation

For many years the major solvent used in the preparation of wheat glutenin has been dilute acetic acid originally proposed by Osborne (6). This procedure suffers from two major disadvantages. The most serious is that a large fraction of the flour protein is insoluble in the acetic acid solution. Accordingly this insoluble or residue protein has received considerably less attention than the readily soluble fraction (7-10). The second disadvantage of the Osborne method is that it yields a glutenin fraction that appears to be highly contaminated with gliadin, albumin, and globulin proteins. To illustrate these points, 10 g. of Manitou flour was fractionated by the modified Osborne procedure (11). The acetic acid-soluble glutenin accounted for 10.9% of the total flour protein, and 26.2% remained as insoluble residue after 3 hr. extraction with acetic acid. PAG electrophoresis of the acetic acid-soluble glutenin gave a pattern with relatively high concentrations of bands of high mobility (Fig. 1). These appear to be albumin, globulin, and gliadin impurities representing a significant proportion of total protein.

Amino acid composition of the acetic acid-soluble glutenin (Table I, column a) shows that this preparation has lower lysine, arginine, aspartic acid, threonine, glycine, alanine, and tyrosine contents, and higher glutamic acid, proline, and phenylalanine contents than the purest glutenin preparation obtained by pH precipitation (compare columns a and g, Table I). These differences confirm the electrophoretic results that the acetic acid-soluble glutenin is highly contaminated with other flour proteins.

Extraction with Water-Acetic Acid-Urea-Detergent Solvents

Meredith and Wren (2) introduced the AUC solvent (0.1M acetic acid, 3M urea, and 0.01M CTAB) to studies of wheat protein. Their studies, and work in this laboratory (12), showed that AUC can dissolve as much as 98% of the flour protein of some bread wheats. Duplicate determinations of the extraction efficiency of protein of Manitou flour used in the present study were performed according to the method of Bushuk and Wrigley (12). Because flour was used instead of ground grain, as used by Bushuk and Wrigley (12), only 5 min. homogenization time was employed. An extractability of $93 \pm 1\%$ was obtained. This value was somewhat lower than the value for ground wheat with 1 hr. homogenization (12) but is essentially the same as that reported by Meredith and Wren (2) using 2 min. homogenization for flour.

Identical extractions with solvents comprising 0.1M acetic acid, 3M urea, and either 0.01M Brij 35 [$C_{12}H_{25}(OCH_2CH_2)_2_3OH$ from Calbiochem.] (AUB) or

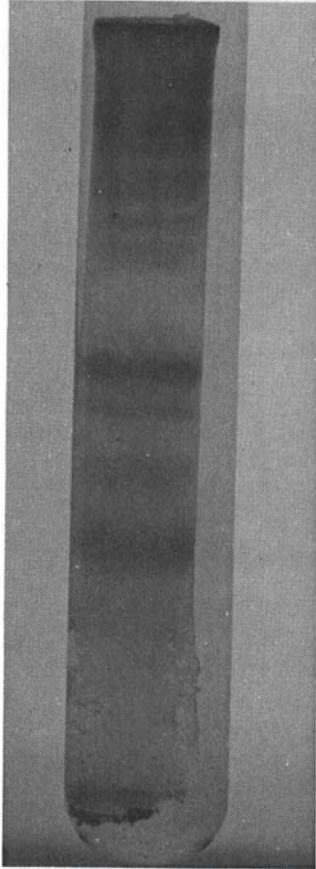


Fig. 1. PAGE pattern of glutenin prepared by the Osborne fractionation.

0.01M sodium dodecyl sulfate removed 89 and 60% of the flour protein, respectively. The AUB extraction was 4 percentage units lower than the AUC extraction. However, Brij 35 is preferred over CTAB for electrophoretic work because it is electrically neutral and therefore does not migrate during electrophoresis.

Figure 2 gives the PAGE patterns of the proteins extracted by AUB and AUC and also shows the effect of dialysis (at room temperature) on AUC extracts. The nondialyzed AUC extract gave a very strongly stained broad band of relatively high mobility. The AUC-dialyzed and AUB-nondialyzed extracts do not show this band. The broad high-mobility band was assumed to be CTAB. These results showed that dialysis for 5 days against distilled water is sufficient to remove all of the free CTAB from the protein solution. Use of the nonpolar Brij 35 as the detergent in the solvent is therefore recommended over CTAB or

TABLE I. AMINO ACID COMPOSITION OF PROTEIN FRACTIONS

	Mole Percent on an Ammonia-Free Basis*						
	a	b	c	d	e	f	g
Lysine	1.41	1.52	1.10	1.04	2.82	0.82	2.19
Histidine	1.71	1.78	1.53	1.90	1.85	1.57	1.81
Arginine	2.43	2.43	2.87	2.14	3.54	2.08	3.01
Aspartic acid	2.85	2.66	2.36	3.32	6.41	2.55	4.42
Threonine	2.91	2.93	2.39	2.13	3.67	2.26	3.01
Serine	6.37	6.68	5.53	5.30	6.81	5.46	6.10
Glutamic acid	35.12	35.72	37.78	36.32	29.73	38.14	30.36
Proline	13.35	12.89	17.06	15.98	5.14	18.10	12.46
Glycine	7.71	7.33	4.80	3.09	6.01	3.02	9.27
Alanine	3.65	3.39	2.96	3.52	5.74	3.07	4.49
Valine	4.31	4.22	3.71	4.70	5.82	4.11	4.66
Methionine	1.23	1.37	1.16	1.31	1.56	1.24	1.43
Isoleucine	3.25	3.26	3.39	4.32	4.70	3.75	3.17
Leucine	6.87	7.02	6.30	7.45	8.56	6.87	7.02
Tyrosine	2.86	2.36	1.77	2.46	2.60	1.75	3.26
Phenylalanine	3.97	4.43	5.30	5.04	5.04	5.20	3.35

a. Osborne glutenin

b,c,d,e. Peaks I → IV Sephadex G-100

f. pH 6.4 70% EtOH soluble protein

g. pH 6.4 70% EtOH insoluble after SE-Sephadex cleanup

*Tryptophan, cysteine (and cystine) were not determined.

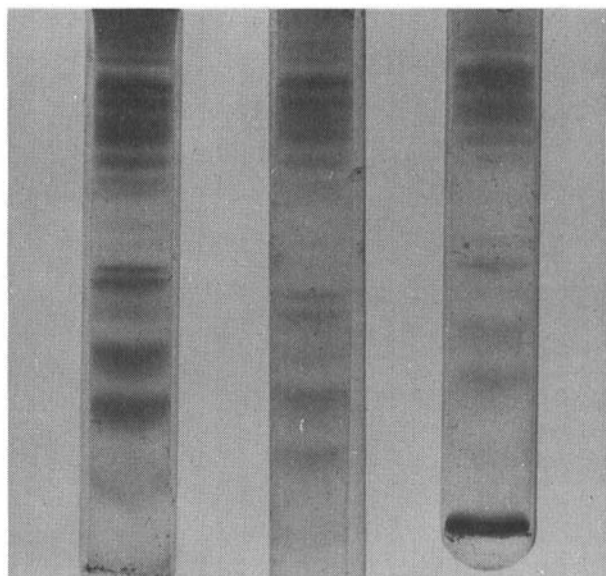


Fig. 2. PAGE patterns of Manitou flour protein dissolved in (left to right) AUB, AUC (dialyzed), and AUC (nondialyzed).

sodium dodecyl sulfate when electrophoresis is planned and lengthy dialysis is impractical.

Comparison of PAGE patterns for proteins extracted using AUC and AUB showed qualitatively similar patterns. Variation in the mobility of some fast-moving proteins solubilized by AUB and AUC can be explained by formation of a detergent-protein complex whose net charge depends upon the charge of the native protein and the detergent involved. Use of uncharged Brij 35 produced a decrease in the mobility of these proteins compared to those complexed with the cationic CTAB.

Gel Filtration

Gel filtration was performed on a 10.0 × 74.0-cm. column of Sephadex G-100 (particle size 40–120 μ) using AUC as the solvent. Upward flow, as recommended by Bushuk and Wrigley (12), allowed 2 months of continuous use of the column at a flow rate of 130 ml. per hr. The eluant was monitored at 280 nm. by an ISCO U.V. recorder and collected at the rate of 12 fractions per hr. Seventy milliliters of AUC-protein solution, clarified by centrifugation at 100,000 g, was applied and chromatographed. The elution profile obtained is shown in Fig. 3. The tubes containing the fractions indicated were pooled and dialyzed against distilled water for 5 days. The dialyzed solutions were freeze-dried.

The electrophoretic patterns in Fig. 4 indicate considerable overlap of chromatographic peaks. Peaks I and II appear similar in their components, although peak II has more of the faster-moving components (R_f 0.3 to 0.6) and more bands in the gliadin region (R_f 0.1 to 0.3). Peak III contains mostly

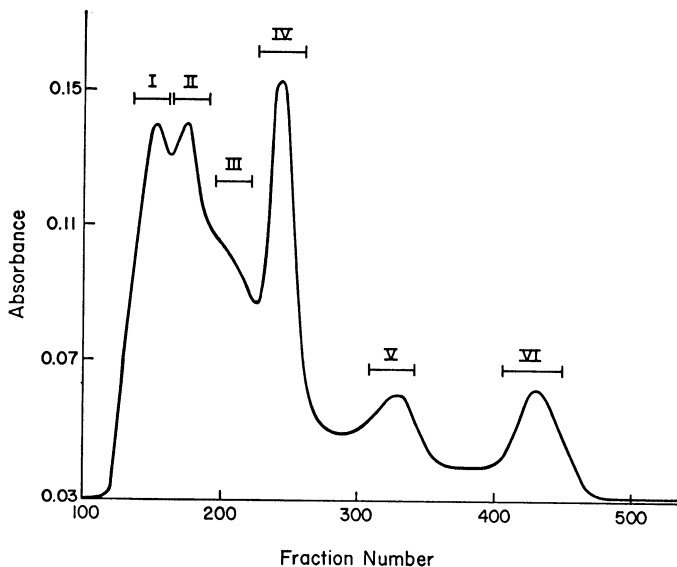


Fig. 3. Sephadex G-100 chromatography of Manitou flour protein in AUC.

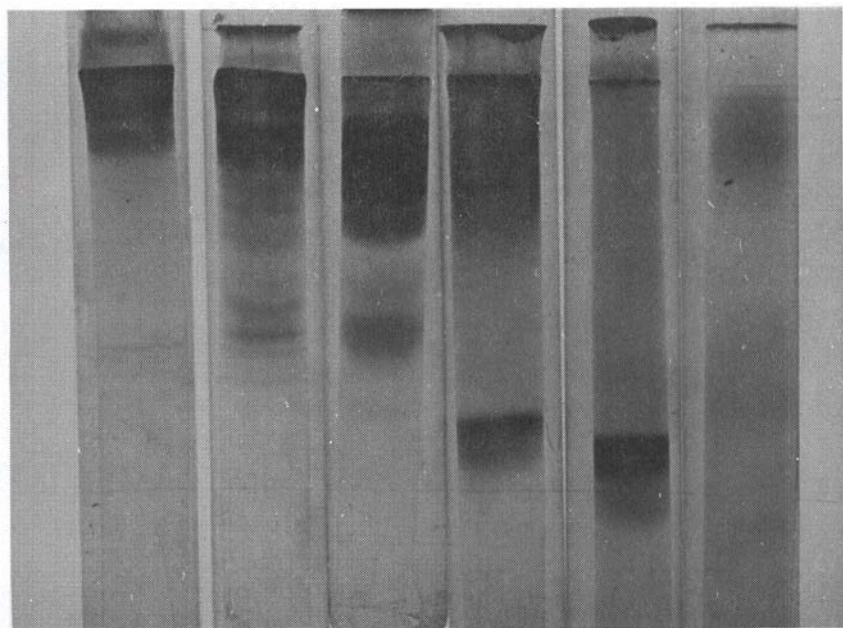


Fig. 4. PAGE patterns of Manitou flour proteins fractionated on Sephadex G-100 using AUC solvent. Left to right, peaks I to VI as in Fig. 3.

gliadins with very little glutenin protein (mobilities between the origin and 0.1). Peak IV contains a significant amount of the gliadins of peak III and a large amount of a fast-moving doublet with an R_f of about 0.7. This doublet is also evident in peak V which contains very little of the slowest-moving components. Peak VI contains a very small amount of protein of high and low mobilities and may represent amino acids and peptides and higher-molecular-weight proteins still eluting at the end of the chromatography.

Amino acid analyses of peaks I to IV confirmed their nature as determined by electrophoresis (Table I, columns b, c, d, and e). The proteins of peak IV are particularly rich in the basic amino acids, arginine and lysine. This explains in part, their high mobility at acid pH (13). These proteins are also very high in aspartic acid but have much less glutamic acid and proline than peaks I, II, or III. Amino acid compositions of proteins represented by peaks I and III correspond to glutenin and gliadin, respectively, when compared with published amino acid compositions (14). Peak II has an amino acid composition that does not fit into either gliadin or glutenin classification but appears to be a mixture of both. The major feature of the proteins of peak I is their very high glutamic acid, proline, and glycine content. Peak III is even higher in glutamic acid and is also characteristic of gliadins having a low glycine and high proline content compared with data for peak I.

pH Precipitation and Ion-Exchange Purification

Gliadins are known to be soluble in 70% aqueous ethanolic solutions whereas

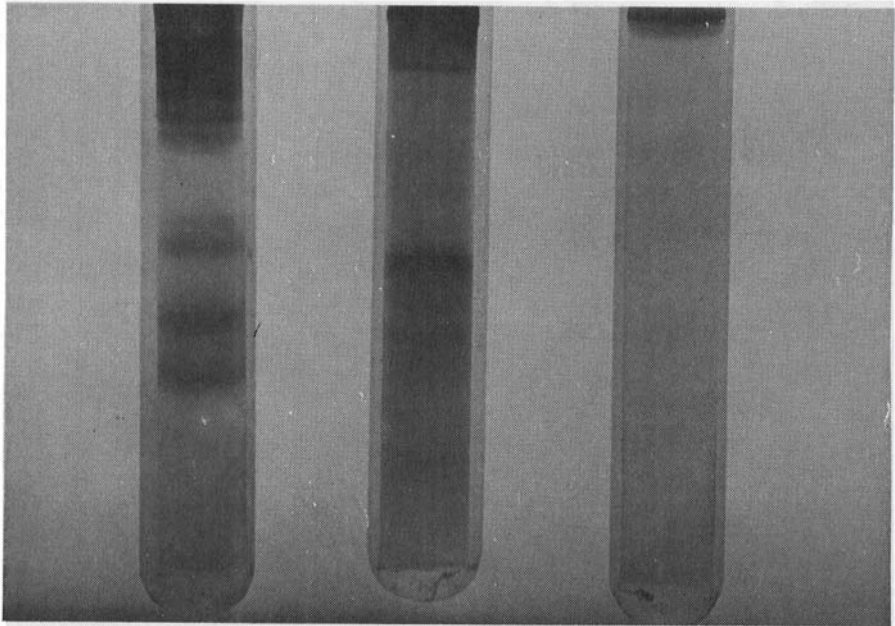


Fig. 5. PAGE patterns of (left to right) pH 6.4 — 70% ethanolic AUC soluble protein, insoluble protein, insoluble protein purified by SE-Sephadex.

glutenins are insoluble in this solvent at pH 6.4 (15). The applicability of this preparative method in conjunction with AUC solvent was tested by the following procedure: Ten grams of flour, in dough form, was washed under a gentle stream of distilled water until a gluten ball was obtained. This gluten was then dissolved in 170 ml. of AUC by overnight magnetic stirring. The solution was centrifuged at 20,000 g for 0.5 hr., and the supernatant was made 70% (v./v.) in ethanol and adjusted to pH 6.4 by the dropwise addition of 1N NaOH. The resulting precipitate was allowed to settle overnight at 2°C. and separated by centrifugation. This precipitate (crude glutenin) was then dispersed in 0.01M acetic acid, dialyzed against distilled water for 5 days, and freeze-dried. An aliquot of the supernatant from the centrifugation after the pH precipitation was also dialyzed and freeze-dried.

The freeze-dried crude glutenin (0.2 g.) was dispersed by overnight magnetic stirring in 50 ml. of AUC containing 0.1 g. of SE-Sephadex C-50 and centrifuged. The supernatant was dialyzed against water and freeze-dried to yield purified glutenin. Ten grams of flour yielded 0.154 g. of purified glutenin representing 10% of the total protein. This unusually low yield resulted because of losses in the purification.

Samples of the protein that dissolved in ethanolic AUC of pH 6.4 and the purified glutenin were subjected to amino acid analysis and PAG electrophoresis. An electrophoretic pattern was also obtained for the initial crude precipitate (see Fig. 5).

The effectiveness of the SE-Sephadex C-50 in removing impurities of low

molecular weight from the crude precipitate is demonstrated strikingly in Fig. 5. No fast-moving bands were evident for this protein, whereas the initial precipitate (center gel) contained a high proportion of gliadin, globulin, and albumin bands. Likewise, the ethanol-soluble protein contained a large number of fast-moving components. Since at pH 3.5 (in AUC) most of the flour proteins would be positively charged, the selective binding of the low-molecular-weight components to SE-Sephadex probably involves molecular sieving action as well as charge. Glutenins, because of their large molecular size, would not penetrate the beads and so would not be adsorbed very strongly. These preliminary results indicate that SE-Sephadex may be extremely useful as a support material for chromatographic separation of flour proteins.

When compared with peak I protein from Sephadex G-100 fractionation (Table I, column b), the SE-Sephadex-purified glutenin is higher in lysine, arginine, aspartic acid, threonine, glycine, alanine, and tyrosine and lower in glutamic acid, proline, and phenylalanine, indicating that it is a purer form of glutenin. Peak I protein was higher in the amino acids that characterize gliadin and lower in those that characterize glutenin when compared with the purified glutenin.

SUMMARY

A modified Osborne fractionation, gel filtration chromatography on Sephadex G-100, and pH precipitation, followed by further purification using an ion exchanger, were compared as preparative methods for glutenin. The third method is recommended as it is simple, effective, and provides pure glutenin in good yield.

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