Further Studies on the Fractionation and Characterization of the Gel Proteins of Wheat Flour by Column Chromatography[1]

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ABSTRACT

The acetic acid-insoluble proteins of wheat flour were extracted with a dilute mercuric chloride solvent and separated into gel-filtered and salt-eluted fractions by agarose chromatography. These fractions were heterogeneous as shown by polyacrylamide gel electrophoresis and appreciable amounts of the salt-eluted proteins did not migrate into the gel. These proteins contained the same amino acids as the wheat flour glutenin fraction with the former containing considerably more glycine and tyrosine than the latter. The electrophoresis pattern of the reduced gel-filtered fraction was different from that of the reduced salt-eluted one.

The wheat flour gel proteins have been shown to affect the dough-mixing optimum of a flour (1). They occur in a highly insoluble state in the hydrated residue obtained when wheat flour is extracted exhaustively with 0.01M acetic acid. Inamine et al. (2) used dimethylaminoethanol to solubilize these proteins and chromatographed them on agarose as well as polyacrylamide using 50% acetic acid-0.15M sodium chloride as eluant. An excluded and a retarded fraction were obtained from the agarose column; analysis by starch gel electrophoresis, however, showed them both to be heterogeneous, and a considerable amount of protein failed to penetrate the gel. The reduced and alkylated gel protein gave an electrophoresis pattern like that of the similarly treated glutenin fraction. Sucrose gradient ultracentrifugation studies were made later on the unreduced proteins solubilized in dilute acetic acid that contained small amounts of mercuric chloride

[1]Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

(3,4). These studies showed that the majority of the solubilized gel proteins had a MW[2] of 44,000 and that some protein resolution had occurred. However, the quantities of fractions obtained were too small to be studied in detail. The authors of the present paper have partially fractionated these gel proteins on a larger scale using a preliminary salt precipitation step followed by a combination of GPC and salt gradient elution. Also, the elutions have been accomplished with milder solvents than those employed by the previous workers (2). This procedure and a preliminary analysis of these protein fractions by PGUE are described in this paper.

MATERIALS AND METHODS

An unbleached flour milled commercially from a mixture of Kansas hard red winter wheat was used to obtain samples of crude gel by a method described in a previous paper (2). Mercuric chloride and CTAB were obtained from Baker Chemical Co. CTAB was recrystallized from absolute ethanol before use. Urea (Allied Chemical Co.) was freed of all carbohydrate material and ions by treating an 8M urea solution with charcoal and passing it through a mixed bed resin (AG 501-X8, 25-50 mesh, Bio-Rad Labs., Richmond, California). Final pH of the urea solution was 7.0. SDS was recystallized from absolute ethanol.

Extraction of Proteins from Gel Solids

Lyophilized gel solids (4 g. containing 20% protein) were extracted with 250 ml. of the appropriate solvent for 2-hr. periods with gentle stirring at room temperature. Solvents used for protein extraction from the gel were 0.01M acetic acid-2 $\times$ 10^{-4}M mercuric chloride and 0.01M CTAB-0.001M hydrochloric acid. The mixtures were then centrifuged at 34,000 $\times$ g at 4°C. for 30 min., the supernatants decanted, and the residue further extracted until no more protein could be solubilized. The protein extracted with mercuric chloride was freed of solvent by dialysis in the manner described previously (3). To remove residual carbohydrate from the protein, 0.2 g. of protein was dispersed in 150 ml. of mercuric chloride-acetic acid solvent and 50 ml. of saturated potassium chloride was added. The resulting protein precipitate was removed by centrifugation and the supernatant was treated with an additional 15 ml. of saturated potassium chloride and centrifuged once more. Finally, the precipitates were combined, redispersed in the mercuric chloride solvent, dialyzed, and lyophilized. Ninety percent of the starting protein could be precipitated in this way, leaving practically all of the carbohydrate in the supernatant.

Protein Analysis

Protein was determined in the crude gel samples by the Kjeldahl method (protein = N $\times$ 5.7); in the mercuric chloride-acetic acid extract by the Lowry method (5) and in the CTAB extract by the use of ninhydrin (6). The protein standard used was purified α-gliadin.

Carbohydrate Analysis

Total carbohydrate was determined in the mercuric chloride-acetic acid extract by the phenol-sulfuric acid procedure (7) and in the CTAB extract by the orcinol procedure (8). Xylose was used as the standard.

[2]Abbreviations used throughout this paper are molecular weight (MW); gel permeation chromatography (GPC); polyacrylamide gel urea electrophoresis (PGUE); cetyl trimethylammonium bromide (CTAB); sodium dodecylsulfate (SDS).

Electrophoretic Analysis

Free boundary electrophoresis of protein solutions was carried out in a Perkin Elmer model 38A instrument, equipped with a cylindrical Schlieren lens system. Horizontal electrophoresis (PGUE) was conducted in 0.017M aluminum lactate-4M urea (pH 3.5) using polyacrylamide gel slabs (27 cm. X 12 cm. X 0.6 cm.) (4). Gels were stained with Amido Black or Coomassie Blue (9).

Reduction of Proteins

Protein fractions were reduced according to the method of Koenig et al. (10) and horizontal polyacrylamide gel electrophoresis was carried out on these products in 0.125M Tris-borate buffer containing 0.1% SDS. The following proteins (with source and MW in parentheses) were used as MW markers: ribonuclease (Pharmacia , 13,700), ovalbumin (Pharmacia , 43,000), bovine serum albumin (Schwarz/Mann , 68,000). Alkylation of the reduced fractions was not done since Woychik et al. (11) found that the reduced fractions before and after alkylation showed the same electrophoretic gel patterns. MWs of the reduced fractions were calculated according to the procedure of Shapiro et al. (12).

Gel Filtration

Sepharose-2B Beads (Pharmacia) were washed free of all fines in water and equilibrated in 4M urea-0.03M acetic acid-0.01M hydrochloric acid. They were then packed into a glass column (98 X 2.5 cm.) and washed with the above solvent until the soluble agarose in the eluate was reduced to a very low level as shown by the phenol-sulfuric acid reagent (7). Samples of gel protein (300 mg. in approximately 3% solutions) that had been extracted with dilute mercuric chloride and freed of carbohydrate by salt precipitation were dissolved by stirring in the above solvent overnight. Chromatography was carried out at room temperature until the retarded fraction had emerged. A linear sodium chloride gradient from 0.100 to 0.105M in the above solvent was then applied to elute that portion of the protein held by ionic binding. Column effluents were collected in 7-ml. fractions and absorbances were measured at 280 nm. Eluates shown in the absorption curves were combined, dialyzed exhaustively against 0.01M acetic acid, and lyophilized before use. Sample recoveries were calculated on the weight of lyophilized solids.

Preparation of Glutenin

Glutenin was prepared by the method described by Huebner (13). The gluten fraction was dispersed in 0.1M acetic acid-2M urea and chromatographed on Sephadex G-100. The first peak that emerged was taken as the glutenin fraction.

TABLE I. PERCENTAGE OF GEL PROTEIN AND CARBOHYDRATE EXTRACTED BY VARIOUS SOLVENTS

Extracting Solvent	% of Total Gel Protein Extracted	% Composition of Solid[a]	
		Protein	Carbohydrate[b]
CTAB, 0.01M - HCl, 0.001M	86	96.0	4.0
$HgCl_2$, 2×10^{-4}M - HOAc, 0.01M	87	97.4	2.6

[a]Values are reported on dry basis.

[b]Values are reported as % anhydroxylose.

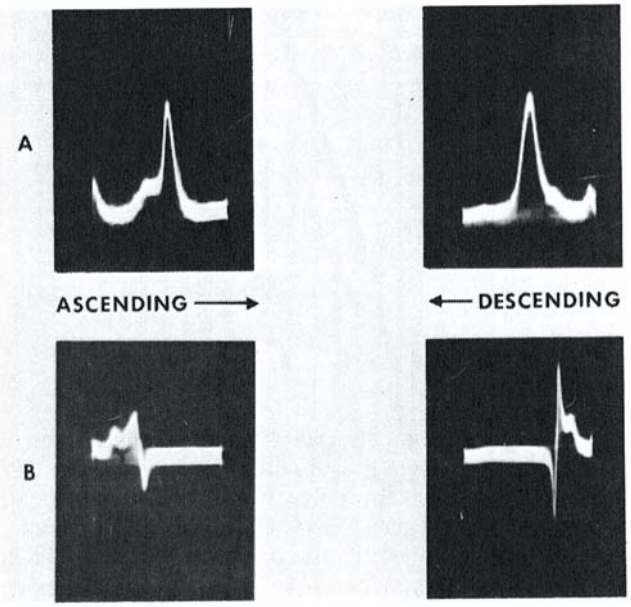

Fig. 1. Moving boundary electrophoresis pattern of gel protein: A, 1% protein in 2 × 10^{-4}M mercuric chloride-0.017M aluminum lactate, conductivity 0.56 mmho. B, 0.5% protein in 0.01M CTAB-0.017M aluminum lactate 0.87 mmho.; 4.5 to 5.0 ma., 25°C., pH 3.5, time of run: in CTAB solvent, 19 min.; in $HgCl_2$ solvent, 56 min.

Amino Acid Analysis

The analysis was carried out with a Phoenix amino acid analyzer (model K8000 B). Proteins were hydrolyzed at 110°C. for 22 hr. (14). No correction was made for loss of amino acids during hydrolysis.

RESULTS AND DISCUSSION

The cationic detergent CTAB solubilized proteins from the gel solids as efficiently as did the mercuric chloride solvent (see Table I). Meredith and Wren (15) found that solvents containing CTAB were capable of overcoming the associative effects among the wheat flour proteins and could be used to extract proteins from flour in nearly quantitative yield. In this work we found that about 14% of the gel protein could not be extracted with this solvent. The Tiselius patterns in Fig. 1 showed that a major and a minor component were extracted by both dilute mercuric chloride and CTAB (the inverted peak that occurs in the lower patterns of the figure is due to CTAB alone). The appearance of two peaks in the moving boundary experiments implies that the two solvents either affected the gel protein in the same way or that they had no effect on the nature of these proteins. Protein resolution by this technique was not adequate to deduce anything further from these pictures.

Heller (16) found that CTAB could be used to extract light-sensitive proteins from animal tissues in a relatively undenatured state. However, we found that the

TABLE II. AMINO ACID COMPOSITION OF GEL PROTEIN AND GLUTENIN

Amino Acid[a]	Gel Protein	Glutenin	Amino Acid	Gel Protein	Glutenin
Lys	7	8	Gly	69	32
His	12	12	Ala	25	24
Arg	18	16	HCys	13	13
Asp	14	19	Val	31	31
Thr	22	19	Met	10	10
Ser	48	43	Ileu	23	30
Glu	272	300	Leu	50	53
Pro	107	132	Tyr	24	14
			Phe	24	39

[a]Reported in mmoles amino acid per 16 g. nitrogen.

use of this detergent as a protein extractant had limitations, because it was difficult to remove CTAB completely from the protein by dialysis. Protein-CTAB complexes could not be studied effectively by polyacrylamide gel electrophoresis, since we could find no dye that would selectively stain the protein and not the detergent. For these reasons we discontinued the use of CTAB and chose the dilute mercuric chloride solvent for protein extractant. Once the mercuric chloride was dialyzed away from the gel proteins and the latter were lyophilized, they were soluble in the urea solvent used in agarose chromatography.

Inamine et al. (2) believed the small amount of xylose-containing polymer that always accompanied the gel protein into solution was covalently bound to the protein. However, our evidence suggests the contrary. Even though both the CTAB and mercuric chloride solubilized this carbohydrate with the protein, virtually all of the carbohydrate could be removed from the protein in a salt-precipitation step, showing that this xylose polymer was not covalently bound to the protein. PGUE

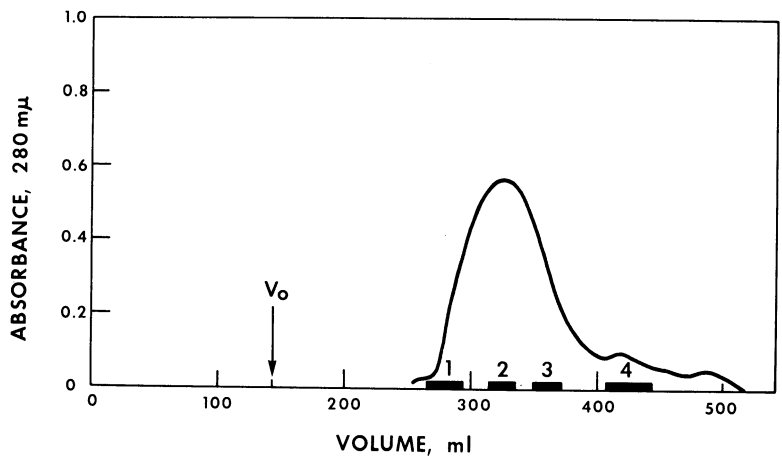

Fig. 2. Elution profiles obtained in the agarose chromatography of the gel protein in 4M urea-0.03M acetic acid-0.01M hydrochloric acid, pH 3.0. Heavy line on the abscissa indicates combined fractions.

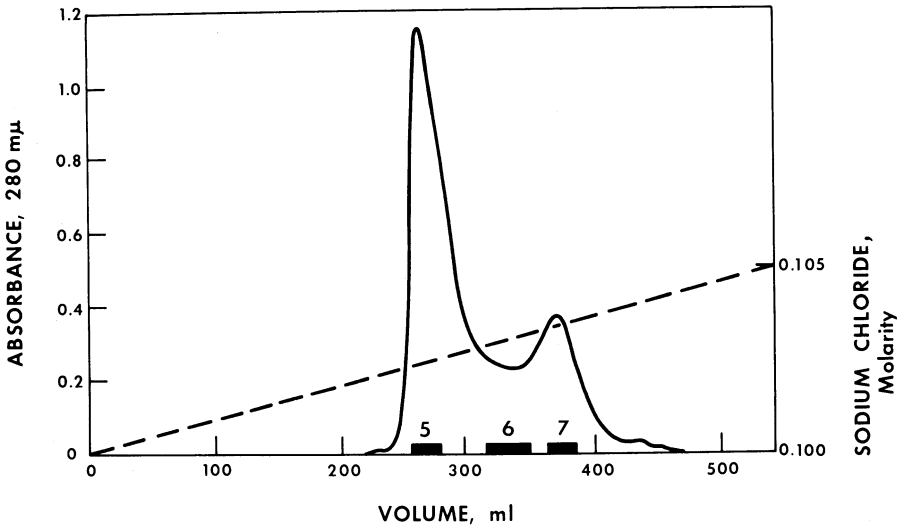

Fig. 3. Elution profile in the elution of gel protein with gradient from 0.100 to 0.105M sodium chloride in 4M urea-0.03M acetic acid-0.01M hydrochloric acid, pH 3.0.

patterns of the gel proteins were similar to those of Inamine et al. (2) in that the latter resembled patterns obtained for the glutenin fraction; i.e., sizable amounts of protein did not move into the polyacrylamide gel, and the fastest-moving components moved no farther from the origin than the γ-gliadins of wheat flour (17). Qualitatively and, to a considerable extent, quantitatively, the amino acid content was like that of glutenin (see Table II). One exception was the low glycine content of the glutenin compared to other values reported in the literature (18) for similar preparations and no reason for this difference is apparent. Also our gel protein was somewhat higher in tyrosine and lower in glutamic acid, phenylalanine, and proline as compared to glutenin. The gel proteins did not resemble the acetic acid-insoluble gluten proteins studied by Cluskey and Dimler (18) in that the preparation of these workers had an amino acid composition resembling that of the water-soluble proteins. The fraction they studied was electrophoretically like glutenin but with additional fast-moving components.

To fractionate the mercuric chloride solubilized gel proteins, agarose column chromatograph of solutions in 4M urea-0.03M HOAc-0.01 HCl, pH 3.0 was used. Platt and Kasarda (19) used a similar solvent to fractionate α-gliadin. Also the experiments were conducted in urea at pH 3.0 or greater, since Huebner and Wall (20) found that 8M urea solutions were unstable below this pH.

Initial agarose GPC runs of the gel proteins showed that about 25% of the original protein loaded on the column was eluted with above solvent (see Fig. 2). An ionically bound portion (which was approximately 50% of the protein originally loaded on the column) could be removed by dilute salt solution (see Fig. 3). (This property was used to fractionate the gel proteins further.) The remaining 25% was bound and not eluted with any solvent tried.

The preparation of agarose used in the work had ion-exchange properties because of the presence of sulfate groups. Conceivably a fraction of these gel proteins was

so salt-sensitive that it precipitated on the agarose at the moment of contact with the ionic groups. This explanation could account for the irreversibly absorbed protein fraction that could not be eluted from agarose with high concentrations of urea or salt. Further evidence that the sulfate groups bring about irreversible protein binding is shown by earlier GPC experiments using mercuric chloride protein on Sephadex G-200 (4). This molecular sieve does not contain ionic groups, and a nearly quantitative recovery of gel protein was achieved from chromatographic columns containing this material. In the present work, GPC on agarose gave the profile of the gel protein fraction shown in Fig. 2.

When the eluted material was subdivided into fractions that were examined by PGUE, heterogeneity was observed in all but one (see fraction 1 in electrophoresis pattern, Fig. 4). A single slow-moving component had separated in the early part of the elution profile. A similar component was also fractionated on a much smaller scale in earlier work by sucrose gradient ultracentrifugation (4). Components 1 through 4 in the peak obtained from agarose by GPC did not have the rubbery glutenin-like characteristics, and there was little high MW glutenin-like material in the peak as evidenced by the lack of protein remaining in the slot of the polyacrylamide gel.

Aside from the resolved protein in fraction 1, there seemed to be little fractionation in the peak obtained by GPC, since the remainder of the protein in fractions 2 through 4 migrated as one wide band with the same mobility. That the

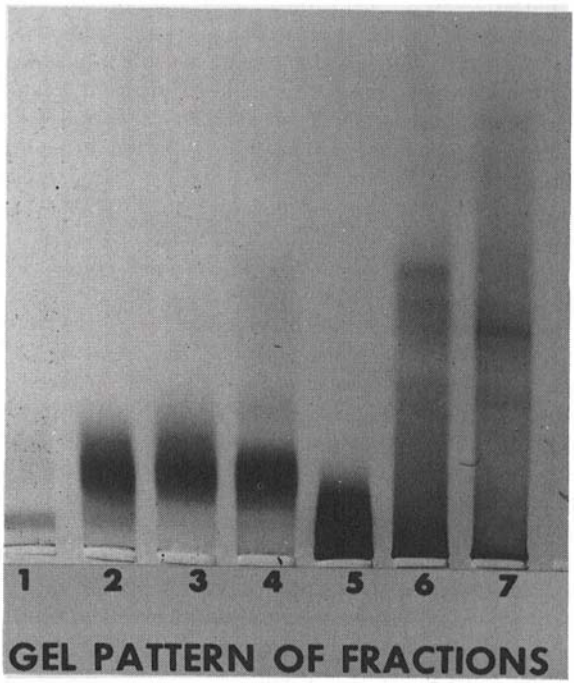

Fig. 4. PGUE patterns of protein fractions obtained from GPC peak (Fig. 2) and salt-eluted peaks (Fig. 3).

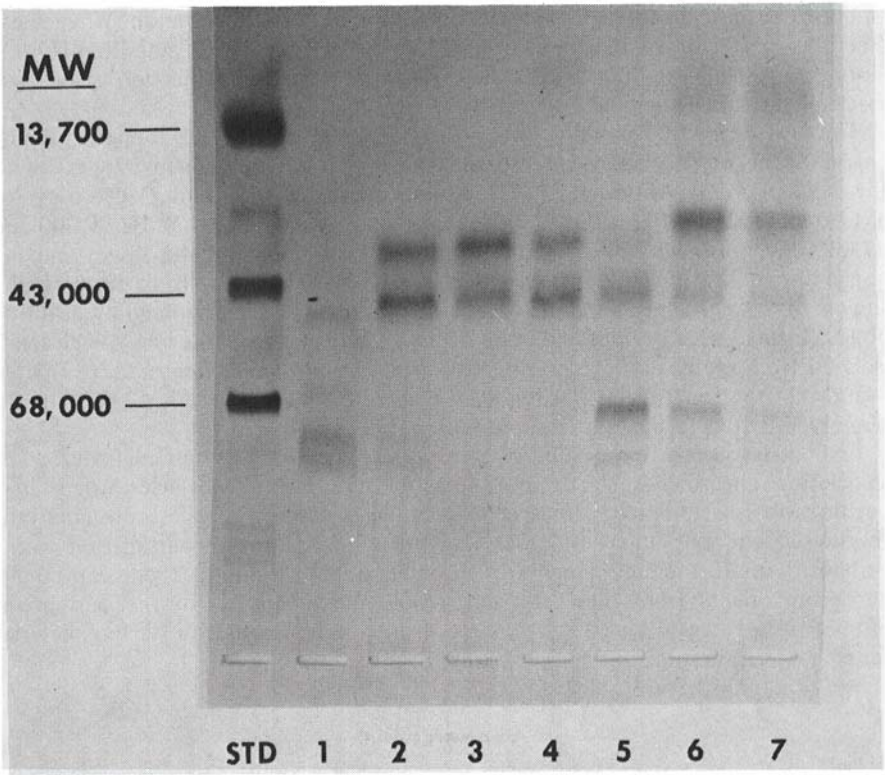

Fig. 5. SDS polyacrylamide gel pattern of subunits of protein fractions obtained by agarose GPC and salt gradient elution. See text for details. MW of reference proteins is indicated.

proteins are the same in these fractions is shown by their respective reduction patterns (see Fig. 5). In each case, the number and mobilities of their subunits are essentially the same. One of the major problems encountered in the fractionation of wheat proteins is that several of the proteins undergo strong interactions with one another. Winzor (21), through use of GPC, demonstrated the occurrence of this interaction in wheat gluten extracts. The gel proteins, studied here, are apparently undergoing interactions similar to those obtained by the above worker in that the same PGUE protein bands appear throughout the latter part of the peak obtained by GPC.

With the salt-eluted fractions (Fig. 3) a considerable amount of protein eluted after the first peak has faster mobilities and is more highly charged than that obtained by GPC. This was expected, since a higher concentration of sodium chloride was required to remove the protein in this peak from the agarose. The entire salt-eluted fraction contained the rubbery glutenin-like material of high MW that remained in the slots during the PGUE runs. It is of interest that this protein possessed sufficient charge to be bound ionically to the agarose. Protein–protein association also occurred in the salt-eluted fractions since PGUE showed these

fractions to have bands and areas of the same mobilities in common. When these fractions were reduced, their gel patterns showed bands of similar mobilities (Fig. 5, slots 5,6,7, MWs 70,000, 85,000, and 46,000), a further indication that these fractions contain associated proteins.

The presence of subunits with MWs 70,000 and 85,000 appears to be characteristic of the salt-eluted proteins and not of those obtained by GPC (with the exception of fraction 1). Fraction 1 was fairly pure, according to electrophoretic criteria, and it reduced chiefly into two subunits of MW 77,000 and 87,000. Even though the protein migrated in the region of the slowly moving ω-gliadins, it did not have the accepted gliadin-like structure, since these results show that it contained subunits of separate polypeptide chains joined by disulfide bonds. Instead, this protein had more of the characteristics of the low MW glutenin isolated from the gliadin fraction by Beckwith et al. (22). Nielsen et al. (23) later found that the starch gel electrophoresis pattern of their reduced low MW glutenin resembled that of regular glutenin more than that of gliadin.

In conclusion, the properties of these gel proteins, i.e., their high degree of association and similar electrophoretic mobilities, present obstacles when their purification is attempted. However, the initial separation of these proteins into gel-filtered and salt-eluted fractions may be a help in future purification work; perhaps, with further refinements in chromatographic techniques, one can obtain pure components from these fractions. Also, the separation of the fairly pure protein from the gel-filtered fraction could provide representative starting material for characterization in studying the nature of these proteins.

Acknowledgment

We thank Mrs. Amy Noma for performing the amino acid analysis.

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[Received September 18, 1972. Accepted December 22, 1972]