Characterization of the Acetic Acid-Insoluble Fraction of Wheat Gluten Protein[1]

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ABSTRACT

The acetic acid-insoluble protein in wheat gluten was extracted in good yield by using a hydrochloric acid-2-chloroethanol solvent after acetic acid extraction. The isolate was found by moving-boundary electrophoresis to have components with mobilities similar to those of glutenin and faster-moving protein. Starch-gel electrophoresis of the insoluble protein after reduction and alkylation demonstrated several components of high electrophoretic mobility similar to that of reduced water-soluble proteins, and lesser amounts of constituents resembling reduced glutenin. The amino acid composition of the insoluble protein was more like that of water-soluble proteins than like that of glutenin. The protein insoluble in acetic acid appears to be a mixture of high-molecular-weight constituents consisting in part of a variety of polypeptides intermolecularly linked together through disulfide bonds and resembling most closely those of the water-soluble proteins of the wheat flour.

Wheat gluten is separated from a dough ball by washing away the starch and soluble proteins with water. The resulting gluten protein is purified by dissolving it in dilute acetic acid, as outlined by Jones *et al.* (1). Cluskey *et al.* (2) have shown that when wheat proteins are separated from flour, 10–20% of the total protein content remains unaccounted for. This percentage varies with the variety or class of wheat flours studied.

Earlier work at this Laboratory indicated that about one-third of this unaccounted-for protein was removed with the starch and that the other two-thirds was acetic acid-insoluble material not extracted from the gluten ball. Other investigators have also noted this acid-insoluble protein (3–5) but have reported no data concerning its identification. The present investigation was carried out to isolate more completely and identify this component of the gluten protein.

MATERIALS AND METHODS

Gluten was prepared from Wichita HRW wheat flour which had been defatted with dry n-butanol at room temperature. The defatted flour was made into dough and washed as described earlier (2). The wash-water was filtered through nylon bolting cloth to retain any glutenous particles that might be carried with the starch. This residue on the cloth was returned to the gluten ball.

Protein was determined by the Kjeldahl procedure ($N \times 5.7$) for all flour and gluten extracts, as well as for the original flour or gluten sample.

Gluten used in the intrinsic viscosity and starch gel electrophoresis experi-

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ments was from Ponca HRW wheat flour. Kjeldahl nitrogen determination showed that the acetic acid-insoluble residue from the Ponca gluten ball contained 10.84% nitrogen, corresponding to 62% protein.

Most of the flour extractions were carried out in the following manner: 1 g. of defatted Wichita flour contained in a test tube was extracted with 10 ml. of the solvent used. The mixture was stirred mechanically for 16 hr. in a hood. Centrifugation for 15 min. in a Servall high-speed angular-head centrifuge at 2,600 $\times$ *g* followed. The supernatant was decanted into a graduate and the starch residue was again washed with 10 ml. solvent for 30 min. After a second centrifugation, the supernatants were combined, the volume was recorded, and two 1-ml. aliquots were taken for duplicate Kjeldahl analyses.

Moving-boundary electrophoresis was carried out at 1°C. in a Spinco Model H electrophoresis apparatus equipped with a cylindrical schlieren lens system and a Rayleigh interference system. The starch-gel electrophoresis of the proteins was done in a simple horizontal apparatus described by Cluskey (6).

Reduction of the proteins was carried out by a procedure similar to that described by Woychik *et al.* (7). The protein was reduced in 8*M* urea and 0.13*M* phosphate buffer, pH 8.5 with mercaptoethanol (0.06*M*). After the mixture had stood overnight, acrylonitrile (0.1*M*) was added and allowed to react for 1–2 hr. (Cyanoethylation modifies the sulfhydryl groups produced by reduction and prevents their reoxidation.) The solution was then adjusted to approximately 0.1*N* with acetic acid, dialyzed against 0.1*N* acetic acid, and lyophilized to yield the protein.

Amino acid analyses were made on the acetic acid-insoluble and glutenin proteins after acid hydrolysis (8). Amino acid composition was determined in a Phoenix amino acid analyzer K8800.

RESULTS AND DISCUSSION

Acetic Acid-Chloroethanol Dispersions

Since only 87% of the total flour protein was extracted with 0.1*N* acetic acid, efforts were made to find a solvent which would extract the protein better. Chloroethanol, a solvent widely used in protein studies, was used in combination with acetic acid. Low concentrations of this alcohol with acetic acid gave poor and variable extractions. Tschoegl (9) has recommended a high concentration of chloroethanol with various acids as an effective protein dispersant. His report prompted us to try higher concentrations of chloroethanol than we originally used with the 0.1*N* acetic acid. In a series of experiments the levels of chloroethanol in 0.1*N* acetic acid ranged from 15 to 85%. The amounts of flour protein extracted amounted to 70–76% and did not correlate with chloroethanol concentration. None of the dispersants containing chloroethanol were as effective as the 0.1*N* acetic acid alone. An increase in either the concentration of the acetic acid or extraction time produced no marked improvement in the amount of protein extracted. Best results with chloroethanol were obtained in a multiple-extraction experiment

based on 50% chloroethanol-0.1*N* acetic acid. Even then, only 80% of the total protein was extracted after five separate 1-hr. extractions. Evidently addition of chloroethanol reduces the extraction efficiency of acetic acid.

Hydrochloric Acid-Chloroethanol Dispersions

Wheat flour extractions were also made with chloroethanol and hydrochloric acid combinations. In this study the chloroethanol concentration was kept constant, but variations were made in acid normality, temperature, and extraction conditions (Table I). When flour was dispersed in 70% chloroethanol-0.1*N* HCl for 16 hr., almost quantitative extraction (98%) was achieved. In contrast to acetic acid, 0.1*N* HCl alone gave a poorer extraction result. These data support the results of Mangels and Martin (10), who studied the solvent action of organic and inorganic acids for proteins. Table I shows that when the hydrochloric acid normality was reduced to 0.01*N* in 70% chloroethanol, the resulting 78% protein extraction compared closely with the 76% extraction obtained with 0.1*N* acetic acid-70% chloroethanol. Protein extraction efficiency depends on extraction time; however, extractions longer than 16 hr. are not necessary to effect nearly total extraction, 98–99%.

TABLE I

TOTAL FLOUR PROTEIN EXTRACTED WITH 2-CHLOROETHANOL AND HYDROCHLORIC ACID

REAGENT	EXTRACTION TIME	TOTAL PROTEIN
	hr.	%
0.1*N* HCl (pH 0.77)	16	28
70% CH_2ClCH_2OH-0.1*N* HCl	16	98
70% CH_2ClCH_2OH-0.1*N* HCl	8	93
70% CH_2ClCH_2OH-0.1*N* HCl	4	89
70% CH_2ClCH_2OH-0.01*N* HCl	16	78

Gluten Protein

The action of the chloroethanol-HCl solvent on whole gluten was investigated to determine if the protein might be adversely affected. A gluten ball, prepared from the Wichita flour by washing the dough ball with saline water, was stirred with 70% chloroethanol-0.1*N* HCl for 24 hr. at room temperature. Semimicro Kjeldahl analysis showed that 95% of the gluten from the ball was extracted by the solvent. A portion of the extract was dialyzed against 0.003*N* sodium lactate. After dialysis, gluten was precipitated by adjusting the pH to 5.5 with dilute sodium hydroxide. The gluten qualitatively exhibited elastic properties similar to those of a gluten ball prepared by standard procedures. This similarity suggested that no major structural damage had been done to the gluten. No attempt was made to detect small changes which might be reflected in baking properties.

Intrinsic viscosity measurements were made to determine if any acid hydrolysis might have occurred because of the low pH. These viscosity determinations were made on Ponca gluten before and after treatment with

70% chloroethanol-0.1*N* HCl, and also with 50% chloroethanol-0.1*N* acetic acid. The measurements were made at 25°C. in a low-ionic-strength solution, 0.003*M* sodium lactate ($\mu = 0.004$), in order to magnify any change which might have occurred. The intrinsic viscosity of gluten is about twice as large in a low-ionic-strength solvent as in one of high ionic strength (11). The intrinsic viscosity of this gluten (0.42–0.43 dl./g.) was not affected by the chloroethanol-acetic acid dispersant but was lowered to 0.34 dl./g. after treatment with chloroethanol-HCl. These data indicate that some change in size or shape has taken place during the treatment with the stronger dispersant. A mild hydrolysis of some kind may occur, most likely a deamidation reaction. Tschoegl (9) has demonstrated the deamidation of gluten in the presence of hydrochloric acid. Holme and Briggs (12) and Beckwith *et al.* (13) have shown that amide groups function as hydrogen-bonding agents in the intermolecular association of gliadin molecules. Deamidation could, therefore, cause an intrinsic viscosity change.

To obtain a procedure that minimized degradation of protein, a two-step extraction procedure was used. The first step involved acetic acid and followed the procedure of Jones *et al.* (1). The hydrochloric acid-chloroethanol treatment of the acetic acid-insoluble residue comprised the second extraction. By this method, the major amount of the gluten protein was not affected by the stronger reagent. Good extraction results with different combinations of solvents, as evidenced by Kjeldahl analyses, were obtained as shown in Table II.

TABLE II

WHEAT PROTEIN DISTRIBUTION IN EXTRACTS OF FLOUR AND GLUTEN WITH DIFFERENT SOLVENT SYSTEMS
(Protein by Kjeldahl in Wichita flour, 14.0%)

	PROTEIN					
EXPERIMENT	In Water-Solubles	In Starch	In First-Extraction Gluten[a]	In Second-Extraction Gluten[a]	In Residue	Total Recovery
	%	%	%	%	%	%
I	8.3	3.7	73.0 70% CH_2ClCH_2OH- 0.01*N* HOAc	10.9 70% CH_2ClCH_2OH- 0.1*N* HCl	3.0	98.9
II	8.0	4.7	73.2 0.01*N* HOAc	7.9 70% CH_2ClCH_2OH- 0.1*N* HCl	4.1	97.9
III	8.0	4.0	74.5 0.01*N* HOAc	8 50% CH_2ClCH_2OH- 0.1*N* HCl	4.0	98.5

[a] Solvent systems used in the first and second gluten extractions are shown below the percentage proteins recovered.

In experiment I, a gluten ball was extracted for 16 hr. at room temperature with chloroethanol-acetic acid. The residue from this extraction was treated with the HCl-chloroethanol dispersant. In experiments II and III the standard gluten isolation procedure with acetic acid was followed by treatment of the residue with the HCl-chloroethanol solvent. The results indicate

that incorporation of chloroethanol in the first gluten extraction (Table II) is not necessary. It may be noted that with gluten the presence of chloroethanol did not decrease the extraction efficiency of acetic acid as happened with whole flour. In the second extraction, chloroethanol-HCl solvent removes the major amount of the remaining protein. The difference in chloroethanol concentrations between extractants used in experiments II and III does not affect the protein yield. Differences in recovery data are within experimental error.

Moving-Boundary Electrophoresis

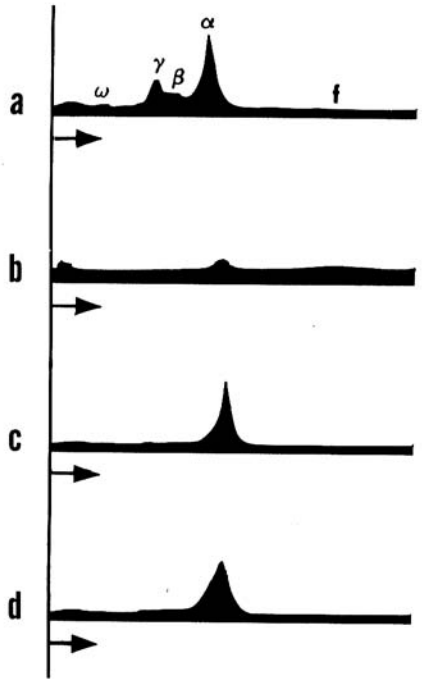
Mobilities of the intact insoluble fraction were determined by moving-boundary electrophoresis and compared with those of other wheat proteins. After extraction (as described above) the proteins were dialyzed against aluminum lactate buffer, pH 3.1, and then subjected to electrophoresis. Figure 1, a, shows a typical pattern of hard winter wheat gluten proteins ($c = 0.72\%$). The gluten was prepared by the procedure of Jones *et al.* (1). The acetic acid-insoluble residue from this procedure was extracted with 50% 2-chloroethanol-0.1*N* HCl and gave the pattern shown in Fig. 1, b. The concentration used here was 0.06%, calculated from the Rayleigh fringe pattern. While this low concentration might prevent detection of minor components, the migration characteristics of the major part of the protein are apparent. The center peak is in the alpha-gliadin or glutenin region. A broad peak is seen in the region of fast-moving material, f.

To establish more completely the gluteninlike character of the material appearing in the alpha-gliadin region, wheat glutenin was treated with 70% 2-chloroethanol-0.1*N* HCl, Fig. 1, c, and 1, d. The main peak in the treated glutenin was broader and appeared in the same position as untreated glutenin and as the material extracted from acetic acid-insoluble fraction. This spreading of the area may be due either to the forming of new charged groups (carboxyl) following deamidation from acid hydrolysis or to a lessening of the intermolecular attraction through hydrogen bonds involving the amide group. Since there appears to be no fast material generated when glutenin is treated (Fig. 1, c and d), one can conclude that the higher-mobility proteins in the acetic acid-insoluble fraction are not glutenin but are more highly charged components related to albumins, or globulins which remained entrapped in the insoluble fraction.

Starch-Gel Electrophoresis

Further characterization of the acetic acid-insoluble protein was carried out by using starch-gel electrophoresis. Figure 2 is a schematic diagram of electrophoretic patterns of glutenin, acetic acid-insoluble protein, and water-solubles, and the alkylated reduced counterpart of each. The three protein fractions were from Ponca wheat, a HRW variety. Concentrations of the dispersions ranged from 4 to 5%.

Electrophoresis patterns a, c, and e (Fig. 2) are from glutenin, acetic acid-insoluble and water-soluble proteins, respectively. The typical pattern for glutenin, a, shows heavy staining at the origin, indicating that the molecules of this fraction are too large to penetrate the gel. Similarly, the acetic



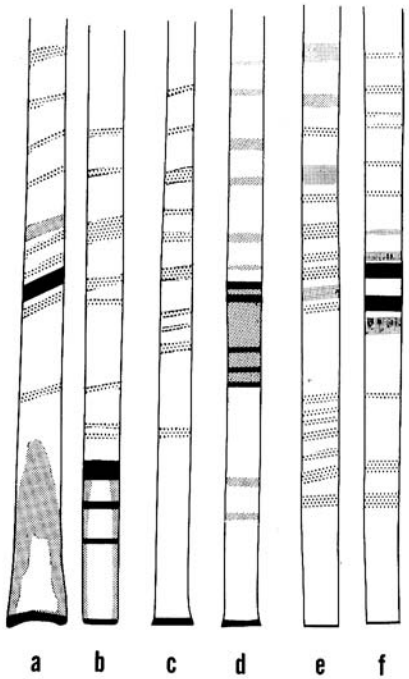


Fig. 1 (left). Electrophoresis patterns of: a, Wichita gluten extracted by 0.01*N* acetic acid (c = 0.72%); b, resulting residue treated with 50% 2-chloroethanol-0.1*N* HCl (c = 0.06%); c, glutenin (Ponca); and d, glutenin (Ponca) treated with 70% 2-chloroethanol-0.1*N* HCl.

Fig. 2 (right). Schematic diagram of starch-gel electrophoresis patterns of Ponca gluten fractions in aluminum lactate buffer, 0.05 μ, 2*M* urea pH 3.2: a, glutenin; b, alkylated-reduced glutenin; c, acetic acid-insoluble protein; d, alkylated-reduced acetic acid-insoluble protein; e, water-solubles; f, alkylated-reduced water-solubles.

acid-insoluble fraction c also did not migrate. These two patterns, a and c, contain traces of fast-moving material. These bands of high mobility resemble some of those in pattern e, the water-solubles. This demonstrates the possibility that water-solubles have been deeply embedded into these protein aggregates and are released during dissolving and electrophoresis. Not all the water-soluble components are present in the acetic acid-insoluble residue, as indicated by comparing the patterns c and e. The indication that certain water-soluble protein types are more readily held to the glutenin is subject to further investigation, for in general all the water-soluble protein should have been removed at this point in the fractionations.

Electrophoresis patterns of the alkylated-reduced protein samples are shown as b, d, and f in Fig. 2. These preparations resulted from reduction and alkylation of the protein isolates after the procedure of Woychik *et al.* (7). Sulfhydryl determinations on samples b and d showed no disulfide links or free sulfhydryl present. In our work, the total acetic acid-insoluble fraction was used, rather than the protein isolated from it by the chloroethanol-HCl method.

In the region of the fast-moving components, the alkylated-reduced insoluble fraction, d (Fig. 2), shows more resemblance to the alkylated-reduced water-solubles, f, than to the alkylated-reduced glutenin, b. It is apparent from the stained origin in b and d that high-molecular-weight material is still present. This material may correspond to large aggregates of peptide chains formed after disulfide cleavage, as recently suggested by Nielsen (14). Each sample was treated similarly; therefore, the question arises whether the acetic acid-insoluble fraction is identical with common glutenin, since the electrophoretic patterns after reduction (b and d) are not alike. In pattern d some components have very high mobilities similar to those of components in the water-soluble protein e. It would appear that the protein in the reduced acetic acid-insoluble fraction is a mixture of polypeptides resembling those of alkylated-reduced water-soluble protein, native water-soluble protein, and a high-molecular-weight component which does not move into the gel.

The amino acid composition of glutenin and acetic acid-insoluble protein in original and reduced-alkylated conditions was determined in a Phoenix amino acid analyzer. Results of the analyses are presented in Table III. The

TABLE III

AMINO ACID CONTENTS OF GLUTENIN AND OF WATER-SOLUBLE AND ACETIC ACID-INSOLUBLE PROTEINS OF WHEAT

(A, mM/16 g. nitrogen; B, percent amino acid residue in wheat protein[a])

	GLUTENIN				ACETIC ACID-INSOLUBLE		WATER-SOLUBLE	
AMINO ACID	A	A[15]	B	B	A	B	A[8]	B
Lysine	9	11	1	1	44	6	28	4
Histidine	11	12	1	2	19	3	23	3
Ammonia	173	219	..	..	123	..	70	..
Arginine	18	17	2	2	39	5	35	4
Aspartic acid	21	21	3	3	53	7	47	6
Threonine	26	24	3	3	36	5	33	4
Serine	56	46	7	6	46	6	48	6
Glutamic acid	288	254	35	33	131	17	144	18
Proline	108	104	13	13	46	6	70	9
Glycine	68	71	8	9	67	9	78	10
Alanine	29	31	4	4	62	8	51	7
1/2 Cystine	12[b]	11	1	1	8[b]	1	45	6
Valine	34	37	4	5	51	7	50	6
Methionine	7	11	1	1	..[c]	..	13	2
Isoleucine	25	26	3	3	36	5	37	5
Leucine	54	52	7	7	64	9	58	7
Tyrosine	22	21	3	3	20	3	19	2
Phenylalanine	28	25	3	3	28	4	15	2

[a] Based on 16 amino acids for acetic acid-insoluble protein (no ammonia or methionine) and 17 amino acids for glutenin and water-soluble protein.

[b] Determined by amperometric titration.

[c] Not determined.

data on reduced-alkylated proteins, not included in the table, were similar to the results on intact proteins. Data for amino acid composition of glutenin and acetic acid-insoluble fractions prepared in the laboratory are compared with literature values for glutenin and water-soluble proteins of wheat. The data are expressed in two ways: (A) mM/16 g. nitrogen, and (B) percent

of amino acid residues in wheat protein. Trytophan was not determined.

Values for the laboratory-prepared glutenin show good agreement with previously reported composition of glutenin (15). Ammonia data are omitted in the percent calculation.

In amino acid composition the data for acetic acid-insoluble protein are more like those of water-soluble wheat proteins than those of glutenin. Several very close agreements are evident. The acetic acid-insoluble fraction is noticeably high in lysine and low in proline and cystine as compared with the water-soluble fraction. On the other hand, the cystine residue result is more like that of glutenin. The arrangement of the polar groups or the amino acid residues in the fractions possibly accounts for the diverse physical characteristics of these proteins.

To summarize, the acetic acid-insoluble protein in wheat gluten has been isolated by using an acidic chloroethanol dispersant. This isolate was shown by moving-boundary electrophoresis to be a gluteninlike species together with a trace of fast-moving material. Additional characterization by starch-gel electrophoresis of the acetic acid-insoluble protein demonstrated 1) its general inability to penetrate the starch gel, indicating a high molecular weight, and 2) the presence of electrophoretically mobile material. Reductive cleavage of disulfide bonds yielded a product similar in electrophoretic behavior to reduced-alkylated water-soluble proteins in starch-gel electrophoresis. The data for amino acid composition, from the acetic acid-insoluble protein, resembled those of water-soluble wheat proteins more closely than those of wheat glutenin.

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