SOLUBILIZATION AND FRACTIONATION OF WHEAT FLOUR PROTEINS INSOLUBLE IN DILUTE ACETIC ACID[1]

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ABSTRACT

The gelatinous residue obtained by exhaustive extraction of flour with 0.01*M* acetic acid and removal of much of the starch by centrifuging appears to consist largely of protein material resembling glutenin. Treatment of the gelatinous residue with 0.1*M* aqueous dimethylaminoethanol solubilized about 85% of the protein; most of the accompanying carbohydrate remained insoluble. The solubilized protein then could be dissolved in 50% acetic acid-0.15*M* sodium chloride, or other solvents with strong protein-dissociating properties, and subjected to gel-filtration chromatography on columns of agarose or polyacrylamide beads. Two high-molecular-weight fractions were obtained. One fraction appeared in the void volume of the agarose column and, like glutenin, did not migrate into a starch gel during electrophoresis. The second fraction required further purification on polyacrylamide beads of smaller pore size. Recovered material gave a trailing pattern upon starch-gel electrophoresis that extended between the origin and a position where the gamma-gliadin band would occur under similar conditions. After the fractions were reduced and alkylated, the electrophoresis patterns were similar to those of reduced and alkylated glutenin, but a few additional faster-moving bands were present in the second fraction.

Earlier papers (1,2) described the highly hydrated residues that are obtained when wheat flours are extracted repeatedly with 0.01*M* acetic acid and pointed out the contrast to the residues of smaller volume and protein content that are obtained from doughs. The observations clearly showed that protein present in the insoluble hydrated residue from flour is converted to an extractable form by dough mixing. Further characterization of the protein in the residue was undertaken. As described in this paper, solubilization and separation from accompanying nonprotein materials was first achieved, with aqueous dimethylaminoethanol (DMAE) as solvent. Alkanolamines have been used previously by Newmark and Myers (3) to solubilize tobacco mosaic virus. The solubilized material then was fractionated by gel filtration on agarose and polyacrylamide beads and compared with gliadin and glutenin by gel electrophoresis.

Materials and Methods

An unbleached commercial hard red spring wheat flour, containing 14.7% protein and 0.44% ash, was defatted with dry n-butyl alcohol (4). Kjeldahl N $\times$ 5.7 was used as the protein content of residue fractions, and no corrections were made for moisture content. Centrifuging was done at 4°C.; all other manipulations were carried out at room temperature.

Extraction of Flour. A portion (500 g.) of the defatted flour was sus-

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pended in 24 liters of 0.01*M* acetic acid. The suspension was stirred gently with a mechanical stirrer for 5 min. and then allowed to settle for 1 hr. The cloudy supernatant was siphoned off. The procedure was repeated until the sample had been extracted six times. The residue was centrifuged at 30,000 $\times$ *g* for 1 hr. Two quite different layers formed. The upper gel layer was carefully removed with a spatula, freeze-dried, and stored at 5°C. The compacted lower starch layer contained only about 0.4% N and was discarded. Thirty-four grams of the dried gel material was obtained from the 500 g. of defatted flour. The dry fraction contained 26% protein and represented about 12% of the protein in the flour prior to lipid removal. In subsequent discussions, this material is designated as whole gel solids.

Extraction of Protein from Gel Fraction. Portions (20 g.) of the whole gel solids were suspended in 1 liter of 0.1*M* DMAE which had been previously deaerated and saturated with nitrogen. The suspension was stirred for 2 min. in a homogenizer (Omnimixer) and then by a magnetic stirrer for 2 hr. in a nitrogen atmosphere. After centrifugation at 14,500 $\times$ *g* for 20 min., the supernatant was decanted and its pH adjusted from 10.0 to 7.5 with 50% acetic acid. The now milky solution was dialyzed for 48 hr. against running distilled water before freeze-drying. Approximately 4.6 g. of material was obtained which contained 16.7% N and represented about 85% of the protein present in the whole gel solids. This material is referred to as DMAE-solubilized protein. For comparative purposes chloroethanol-hydrochloric acid was used as an acidic dispersant (5). This solvent extracted 45% of the whole gel protein.

Gel Filtration. Agarose beads (3%) were prepared according to the procedure of Hjertén (6) except that 1.5 g. of Arlacel 83 (sorbitan sesquioleate, Atlas Chemical Industries, Inc.) was added and a stirrer speed of 2,000 r.p.m. was used to obtain beads of comparable size. "Seakem" agarose was obtained from Bausch & Lomb, Inc. Columns 2 $\times$ 90 cm. and 4 $\times$ 90 cm. were prepared in 50% acetic acid-0.15*M* sodium chloride. The columns were washed with 8 to 10 column volumes of solvent prior to use. Polyacrylamide Bio-Gel P-60 (Bio-Rad Laboratories) was hydrated in the solvent for 24 hr. prior to column preparation.

Samples to be chromatographed were suspended in 50% acetic acid (without sodium chloride) at 5 to 10 mg. per ml. and homogenized for 2 min. (Omnimixer). The mixture was stirred for 24 hr. prior to centrifugation at 30,000 $\times$ *g*. Approximately 6% of the material remained insoluble. Up to 10 ml. of the solution was placed on a 2 $\times$ 90 cm. column; a maximum of 50 ml. was placed on a 4 $\times$ 90 cm. column. Elution was carried out with 50% acetic acid-0.15*M* sodium chloride. The protein was eluted at a flow rate of 12 to 28 ml. per hr. The effluent was collected in 4- to 8-ml. portions by means of a volumetric fraction collector (Gilson). The UV absorption of the effluent was continuously recorded at 280 mμ.

Resolution of the proteins on agarose columns required the presence of salt in the elution buffer. Apparently the agarose preparation contains enough sulfate groups to contribute significant ion-exchange effects.

The agarose columns were used with 50% acetic acid for more than 4 months without any visible signs of deterioration or loss of resolving power.

Samples of the chloroethanol-hydrochloric acid extract of the whole gel solids were fractionated in a similar manner. Glutenin was obtained by the method of Nielsen *et al.* (7); i.e., a 0.3% gluten dispersion in 0.1*M* acetic acid was adjusted to pH 4.5 and 0.05 ionic strength with sodium acetate to precipitate glutenin.

Reduction and Alkylation of Proteins. The various protein fractions were reduced in 8*M* guanidine hydrochloride and 0.1*M* phosphate buffer at pH 8.0. Mercaptoethanol was added to give a 0.3*M* solution and the reduction allowed to proceed for 1 hr. Alkylation then was performed with acrylonitrile (8). A two-molar excess of the reagent was added and reaction was allowed to proceed for 30 min. An equal volume of 50% acetic acid was added to give pH 3.0, and the solution was dialyzed prior to lyophilization.

Starch-Gel Electrophoresis. Horizontal electrophoresis was performed in a water-cooled apparatus. The gels ($28 \times 7 \times 0.6$ cm.) contained a nominal 18% concentration of starch, 7.5*M* urea, and 0.017*M* aluminum lactate buffer of pH 3.1 (4). They were prepared as described by Cole and Mecham (9). Migration was at 300 volts (10 to 12 ma.) for 24 hr. The gels were sliced, as described by Wake and Baldwin (10), and then stained with 0.1% aqueous nigrosine for 20 min. After destaining with 10% glycerol in water, the gels were photographed (panchromatic film, Wratten A filter, back lighting), and dried by the procedure of Wake and Baldwin.

Results

Extraction of Protein from Whole Gel Solids. Several acidic, neutral, and alkaline solutions were tried in attempts to solubilize the protein in the whole gel solids. Few solvents for the protein were found, and when both solubilization of protein and separation of protein from carbohydrate were considered, no other solvent was found to approach the DMAE treatment in effectiveness.

The dried material from whole gel solids solubilized by DMAE and recovered after neutralization and dialysis contained only 2 to 3% carbohydrate *vs.* about 70% in the dried whole gel solids. Acid hydrolysis followed by paper chromatography showed the carbohydrate in the DMAE-solubilized material to be almost entirely xylose, whereas in the whole gel solids the carbohydrate consisted so predominantly of glucose that the xylose could not be detected.

Formic acid (90%) dissolved the whole gel solids completely, but simple procedures to separate the solubilized proteins from carbohydrate were not found. Dilute formic acid was ineffective. A mixture of pyridine:formic acid:water (32:45:23 parts by volume) dissolved about half as much protein as the DMAE treatment; but this was less effective than the chloroethanol-hydrochloric acid mixture used as an acidic solvent to compare with the alkaline DMAE. Concentrated solutions of urea, guanidine hydrochloride, and acetic acid could dissolve most of the protein material after separation from carbohydrate and were employed for fractionation experiments, but were not useful to separate protein from the whole gel solids because they dissolved carbohydrate along with protein.

Gel Filtration. Information on the particle size distribution of DMAE-

solubilized protein was obtained by gel filtration. The insolubility of the protein (and its failure to migrate into starch gel in electrophoretic examination) indicated that particle size would be rather high and that beads of low solids content (high porosity) would probably be required to achieve any separations. Agarose beads were the only type found suitable at low solids content for use in solvents with a strong protein-dissociating tendency. Even at a 3% agarose concentration, beads maintained their size and porosity in 50% acetic acid that also contained guanidine hydrochloride up to 3 to 4*M* concentration. Polyacrylamide beads could be used in such solvents only with a moderately high degree of cross-linking, and so were expected to be useful for fractionation only in a lower molecular weight (MW) range.

A typical elution curve obtained on a 3% agarose-bead column with 50% acetic acid-0.15*M* sodium chloride as solvent is shown in Fig. 1. The elution

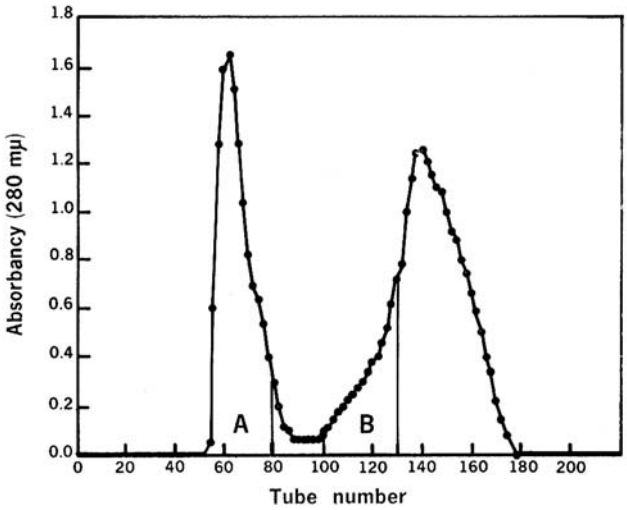

Fig. 1. Fractionation of dimethylaminoethanol-solubilized protein from whole gel solids on 3% agarose column (4 × 90 cm.). Eluted with 50% acetic acid-0.15*M* sodium chloride at 24 to 28 ml. per hr.; 8-ml. fractions.

profile did not change when 50% acetic acid-6*M* urea, 1*M* acetic acid-8*M* urea, or 0.1*M* acetic acid-6*M* guanidine hydrochloride, all containing 0.15*M* sodium chloride, were used. A major portion (fraction A) was large enough in particle size to be excluded from the beads, appearing as soon as the void volume of solvent had passed through the column.

Rechromatography of fraction A on 3% agarose was attempted, but only about half the dried material could now be dissolved even in 50% acetic acid-6*M* guanidine hydrochloride. Once the material was dissolved, guanidine hydrochloride was not required to keep it in solution. The solubilized portion was placed on a 3% agarose column after dilution with 50% acetic acid to give a 3 to 4*M* concentration of guanidine hydrochloride. The dilution step

was used to prevent contraction of the gel column. Elution was conducted with 50% acetic acide-0.15*M* sodium chloride. A typical result from the rechromatography of fraction A is shown in Fig. 2. It is quite evident that additional material of lower particle weight had been dissociated and separated by the

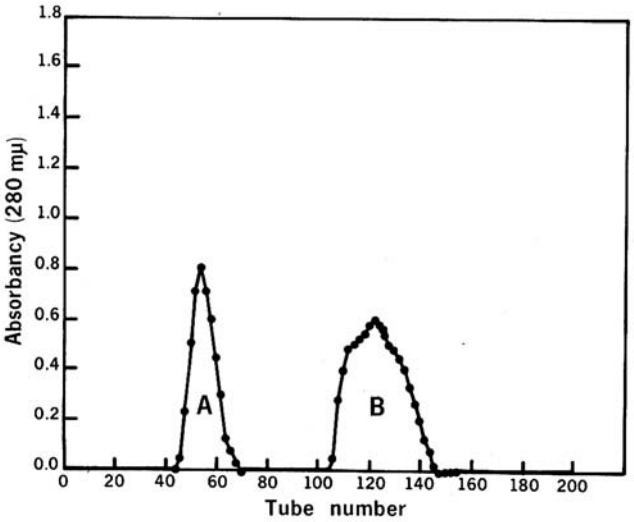

Fig. 2. Rechromatography of fraction A of Fig. 1; solvent and procedure as for Fig. 1.

second treatment. This was eluted with a peak near tube 125 (Fig. 2), appreciably before the peak near tube 140 in Fig. 1. This together with the broadened front of the second peak (Fig. 1) suggested that the tubes from 100 to 130 (fraction B, Fig. 1) be rechromatographed to determine whether two components could be resolved.

Accordingly, fraction B (of Fig. 1) was chromatographed on polyacrylamide beads (Bio-Gel P-60) with a porosity in the absence of protein-dissociating agents useful for fractionations in the MW range of 30,000 to 60,000. As with fraction A, fraction B now required 50% acetic acid-6*M* guanidine hydrochloride for dissolution. It was completely soluble in this solvent, however. The solution was diluted with 50% acetic acid to give a 3 to 4*M* guanidine hydrochloride concentration prior to chromatography. Elution was carried out with 50% acetic acid-0.15*M* sodium chloride. The result of such a fractionation is shown in Fig. 3. Rechromatography of the main portion of the peak gave an elution profile as shown in Fig. 4. Thus, it appears that fraction B (Fig. 1) contains at least two components, but the material represented by the larger part of that peak has not been subjected to further chromatographic examination.

Starch-Gel Electrophoresis. Rechromatographed fraction A (Fig. 2) was found incapable of penetrating the starch gel. This fraction and the material insoluble in 50% acetic acid-6*M* guanidine hydrochloride (after the first 3%

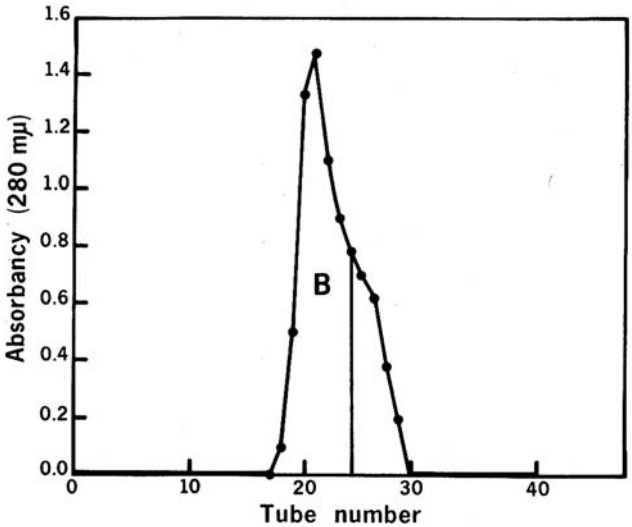

Fig. 3. Fractionation of material in area B of Fig. 1 on polyacrylamide (Bio-Gel P-60). Eluted with 50% acetic acid-0.15*M* sodium chloride at 10 to 12 ml. per hr.; 4-ml. fractions; 2 × 90 cm. column.

agarose fractionation) were therefore reduced and alkylated. Electrophoresis was carried out with similarly reduced and alkylated gliadin and glutenin. The results are shown in Fig. 5, gels 1 to 4. It is evident that the patterns of these fractions, obtained from the acetic acid-insoluble flour protein, are quite similar to the pattern from glutenin. As reported by Woychik *et al.* (11), reduced

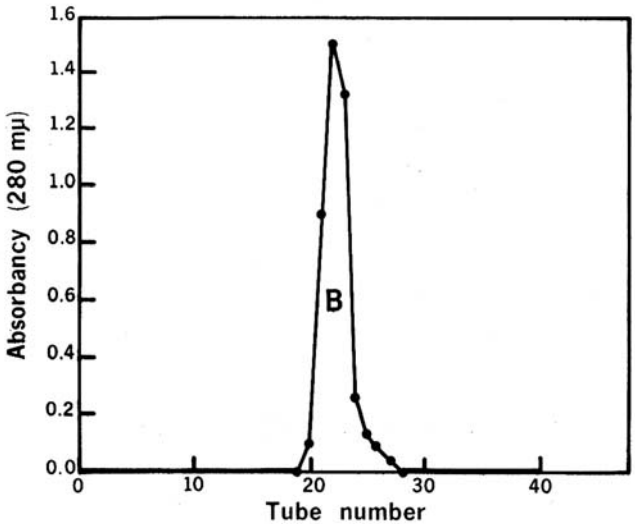

Fig. 4. Rechromatography of material from area B in Fig. 3; solvent and procedure as for Fig. 3.

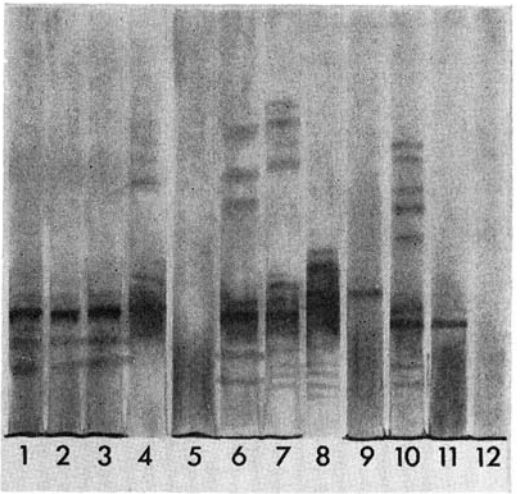

Fig. 5. Starch-gel electrophoresis of fractions obtained by gel filtration, in pH 3.1 aluminum lactate (0.017*M*), 7.5*M* urea. Sample 1, reduced and alkylated glutenin; 2, reduced and alkylated fraction A (Fig. 2); 3, reduced and alkylated Fraction A (insoluble portion from Fig. 1); 4, reduced and alkylated gliadin; 5, fraction B (Fig. 4); 6, reduced and alkylated fraction B; 7, reduced and alkylated gliadin; 8, gliadin. Samples 9 through 12, chloroethanol-hydrochloric acid-treated materials; sample 9, fractionated on 3% agarose, fraction not excluded from gel; 10, reduced and alkylated material as for 9; 11, reduced and alkylated material excluded from agarose gel; 12, as for 11 but not reduced and alkylated.

gliadin and glutenin possess several bands in common, and at least one of the major common bands also is present in the A fraction.

Electrophoretic results for purified fraction B (Fig. 4) are shown in Fig. 5, gels 5 and 6. This fraction in starch gel trails between the origin and the gamma-gliadin region (gliadin, gel 8). Reduced and alkylated fraction B shows identical bands with reduced and alkylated glutenin and fraction A in the region of low mobility of the pherogram. However, there are several bands with mobilities greater than that of gliadin which are observed in reduced and alkylated fraction B. It is interesting to note that Beckwith *et al.* (12) have reported the separation of a high-MW protein from gliadin which trails in starch-gel electrophoresis in a manner similar to that of fraction B.

Starch-gel electrophoretic patterns for fractions obtained from protein extracted from the whole gel solids with chloroethanol-hydrochloric acid are shown in Fig. 5, gels 9 to 12. It is evident that materials comparable to fractions A and B of Fig. 1 were obtained.

The fractions from either DMAE- or chloroethanol-solubilized protein thus appear to be similar to glutenin with respect to the identity of the segments joined by disulfide bonds.

Discussion

The reasons for the efficacy of DMAE in solubilizing the gel protein and in separating it from carbohydrate are not apparent, but DMAE was clearly

superior to any other agent tried. In using DMAE, it was thought necessary to limit the time of exposure to alkaline pH to minimize the possibility of producing artifacts. However, the similarity of the fractions obtained by chloroethanol-hydrochloric acid and by DMAE treatments indicate that exposure to the pH 10 to 10.5 conditions employed with the latter was not harmful. The retention of pentosan by the solubilized protein is in sharp contrast to the removal of hexosan, and suggests that the pentosan may be present in a glycoprotein. However, no other evidence that glutenin contains glycoprotein components has been noted.

Although various concentrations of acetic acid were tried, 50% concentration was found best to solubilize the material extracted with DMAE solution. Concentrated acetic acid has been used to study the dissociation of insulin (13) and the solubilization of ribosomal proteins (14), among others. According to Singer (15), solubilization of proteins by this type of solvent is thought to have effects simultaneously on electrostatic forces, Van der Waals forces, and hydrogen bonding between protein molecules and proteins and solvents.

Agarose gel beads have been used to fractionate high-MW biopolymers, but application to wheat proteins has not been reported previously. The present results indicate that agarose can be used successfully to fractionate high-MW proteins of wheat even though it is necessary to employ solvents with strong dissociating effects: definite separations of material on the basis of particle size clearly were obtained in a reproducible manner. Some association of protein molecules seems to persist at the concentrations used here, as shown especially by the behavior of fraction A, but the observations also give promise that fractions homogeneous in particle size can be separated.

Conclusive results with proteins that are so poorly soluble and thus presumably so resistant to dissociation are difficult to obtain, but perhaps separation of these fractions from flour, rather than from gluten, provides a better opportunity to obtain homogeneous fractions, because the various effects of dough mixing, e.g., on –SH and S–S bonds, are avoided (16).

Otherwise, the fractions obtained from the whole gel solids appear to be much like glutenin. Certainly the reduced and alkylated fractions show basically the same bands after starch-gel electrophoresis as does glutenin similarly treated. Also, when gluten itself is extracted with dilute acetic acid, approximately 10% of the protein remains insoluble. Cluskey *et al.* (17) have studied this fraction and reported it to be a gluteninlike protein species of high molecular weight. Detailed chemical and physical comparisons of the gluten and flour proteins insoluble in dilute acid will be required to determine whether they are basically different.

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