IMPROVED CHROMATOGRAPHIC SEPARATION OF GLIADIN PROTEINS ON SULFOETHYL CELLULOSE[1]

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ABSTRACT

A new procedure of ion-exchange chromatography has been developed that gives better resolution of individual proteins from Ponca wheat gliadin as measured by starch-gel electrophoresis of the separated fractions. The system is based on a 2.8 × 50-cm. column of sulfoethyl cellulose, a strong cation exchanger effective at low pH. The pH 3.6 buffer consisting of 0.03*M* acetic acid and 0.01*M* HCl, used for application and elution of protein, contained 8*M* urea. The ionic strength of the eluting buffer was gradually increased by an apparatus producing a variable gradient from 0 to 0.15*M* sodium chloride. These optimum conditions were arrived at after tests of other ion-exchange materials, solvents, pH values, and ionic gradients. The final method yielded some pure individual gliadin proteins directly and also others which required minimum repurification.

Isolation of individual wheat gliadin proteins is essential for structural determinations that can lead to further understanding of protein properties. Since the amino acid contents and molecular sizes of these proteins appear similar (1,2) and since the proteins are insoluble in neutral solutions, separation of gliadin into homogeneous proteins has not been readily achieved. Numerous methods were investigated, including ion-exchange chromatography (3–5), gel filtration (6), paper curtain electrophoresis (7), and solvent fractionation (8). With the development of improved criteria for homogeneity, the separations obtained with each of these methods have been found incomplete. Whereas moving-boundary electrophoresis distinguished only four components in gliadin, starch-gel electrophoresis, at present the best procedure, established that wheat gliadin contains more than eight components (9).

[1] Manuscript received August 5, 1965. Presented at the 50th annual meeting, Kansas City, Mo., April 1965. Contribution from the Northern Regional Research Laboratory, Peoria, Illinois. This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Mention of firm names or trade products does not constitute endorsement or rejection by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Chromatography on ion-exchange cellulose has been the most extensively used method for the separation of gliadin. Woychik *et al.* (3) first fractionated whole gluten on carboxymethyl cellulose (CMC) using a stepwise reduction in pH to effect resolution. The gliadin was separated into four fractions which differed in mobilities on moving-boundary electrophoresis. Simmonds and Winzor (4) chromatographed the acetic acid-soluble proteins of wheat flour on CMC columns in 1*M* *N*,*N*-dimethylformamide (DMF) solutions using an increasing ionic strength gradient of NaCl to remove the protein. This procedure gave a greater number of peaks of eluted protein. When Wrigley (5) used a similar system, starch-gel electrophoresis revealed that each peak contained more than one protein species.

Additional methods of fractionation have been used to supplement the separation achieved by ion exchange. Woychik and Huebner (7) used continuous-flow paper electrophoresis to prepare gamma-gliadin free of contaminating beta components present in fractions from CMC columns. This method, while yielding pure protein, was limited by the small amount of protein that could be separated and by the long time that it required. Elution of purified components from starch gels after electrophoresis also yielded amounts too small for extensive study (10). The alpha fraction has been further purified by removal of the higher-molecular-weight (MW) contaminants on Sephadex G-75 or G-100 columns (11).

This study was undertaken to establish whether column chromatography on cellulose ion exchangers exhibited further potential for providing larger quantities of pure proteins. A wide range of variables involved in chromatography of proteins were investigated, such as different ion exchangers, solvents, pH, ionic strengths, and column dimensions. Information was obtained that adsorption and aggregation phenomena complicated chromatography. Efforts to overcome these factors resulted in an improved system for chromatography on sulfoethyl cellulose (SEC) that separated individual gliadin proteins better.

Materials and Methods

Gliadin was prepared from Ponca wheat flour by acetic acid extraction of the gluten and fractionation by solubility in neutral 70% ethanol as described previously (12). High-MW components in the gliadin were eliminated by chromatography on Sephadex G-100 according to the method of Beckwith *et al.* (11). In this work only the last peak was used, that is, the lower-MW fraction eluting from the Sephadex column and containing the alpha, beta, and gamma com-

ponents plus some water-soluble proteins. This protein contained 8% moisture when equilibrated with room atmosphere, and a 1% solution had an absorbance of 5.75 at 280 mμ. On a moisture-free basis it contained 17.5% nitrogen.

As previously described (3), CMC from Hercules Powder Company was treated preliminary to use in ion-exchange columns. Phosphonic-acid cellulose (PAC) and SEC were obtained from Bio-Rad Laboratories. The last two modified celluloses were suspended in 3 volumes of water, the coarse particles were allowed to settle, and the fines decanted. This process was repeated three times to give a product that had a suitable flow rate in long columns. The cellulose was then equilibrated with the starting buffer, and the columns were packed in glass tubes of appropriate dimensions (4.0 × 20, 2.8 × 50, and 2.8 ×100 cm.) terminated with fritted disks or glass-wool plugs.

DMF was used as obtained from Matheson, Coleman, and Bell. Baker analyzed-grade urea was further purified before use by dissolving it in dilute acetic acid and then passing it through a short SEC column, which eliminated ammonia and some other impurities. This purification lowered the blank absorbance of the buffer and prevented impurities from accumulating on the chromatographic column.

A Varigrad device (13) from Buchler Instruments was used to attain various gradients of ionic strength in the eluting buffer. The device consisted of nine 400-ml.-capacity chambers connected in series and provided with stirrers. The concentration of sodium chloride in the buffer solution placed in each chamber was selected to give the desired gradient in accordance with tables prepared by Peterson and Sober (13). By means of the Varigrad the sodium chloride content of the eluant was altered in various gradients from 0 to 0.03*M* initially to 0.1 to 0.3*M* at the end of the run.

Samples of 400 mg. protein of 8% moisture were applied to the columns in approximately 2% solutions. The flow rates of the columns were approximately 10 ml./hr./cm.[2] Acetate buffers maintained the pH of the elution solvents. The buffers most generally used were 0.05*M* acetic acid-0.005*M* sodium acetate, pH 4.0; 0.05*M* acetate acid-0.02*M* HCl, pH 2.0; and 0.03*M* acetic acid-0.01*M* HCl, pH 3.6. When either urea or DMF was added, the pH of the buffer changed; the measured pH of these solutions is given in the results. All chromatographic separations described in this paper were run at room temperature. A few tests of separation on SEC were made at elevated temperature (50°C.), with little improvement if any in the results. This contrasts with the finding of Wrigley (5) that elevated temperatures give better separation on CMC.

The column effluent was monitored for protein content by a Canalco automatic recording analyzer that measured approximate absorbance of the solution at 280 mμ. The effluent was collected in 20-ml. fractions in test tubes by an automatic fraction collector. For more precise measurement of protein content the solution in the tubes was analyzed for absorbance at 280 mμ in 1-cm. silica cells with a Beckman DU spectrophotometer. The tubes under a single absorbance peak were combined and dialyzed against 0.01*M* acetic acid to remove the solvents and salts from the protein solutions before lyophilization. Recovery of the protein was calculated both by absorption of the eluted peaks at 280 mμ and by weight of recovered proteins equilibrated at room conditions. Elution was continued until recovery of protein was 95 $\pm$ 5% by both criteria.

Starch-gel electrophoresis of each fraction was conducted in 0.025*M* aluminum lactate buffer-3*M* urea at pH 3.1 according to the method of Woychik *et al.* (9) as modified by Beckwith *et al.* (11).

Experimental Work and Results

Several possible procedures exist for elution of proteins from cellulose exchangers. The buffer may be changed either in pH or in ionic strength, or a combination of both. The changes may be stepwise or continuously gradient. The initial procedure of Woychik *et al.* (3) for chromatography of gliadin on CMC columns consisted of stepwise elution with a series of four buffers, each of lower pH. The gel-electrophoresis patterns of the eluted proteins in Fig. 1 demonstrate that

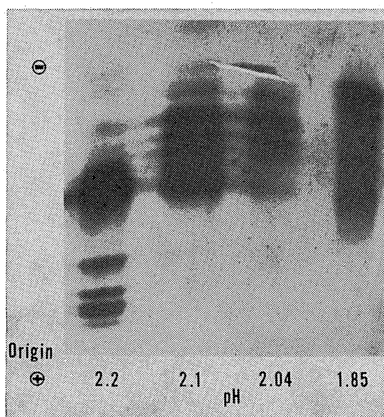

Fig. 1. Starch-gel electrophoretic pattern of chromatographically separated fractions of gliadin on carboxymethyl cellulose (CMC). Stepwise elution with 0.01*N* acetic acid and various concentrations of HCl resulted in fractions indicated at designated pH.

while fractionation into related groups of proteins occurs with the method, separation is not complete. Although gradient reduction of pH spread over a greater volume of eluant gave somewhat better resolution, the procedure still was not satisfactory.

In the method of Simmonds and Winzor (4), elution from CMC was conducted at constant pH with an increasing gradient of sodium chloride. To ensure solubilization of the protein at higher ionic strengths, 1*M* DMF was incorporated into the buffer solution. This gradient ionic-strength elution technique with a constant pH buffer containing solubilizing agents was used in all subsequent experiments.

The most extensively used cationic exchange material in gluten protein work, CMC, has decreased effectiveness at very low pH values because of the weak acid nature of its functional groups. Because gliadin proteins have a low content of basic amino acids and little difference in apparent charge, it was thought that the best separation could be obtained at pH values below 4, which is near the effective limit of CMC. Tests were therefore made with separations on the strong cation exchangers SEC and PAC, which are effective at lower pH values.

The separation on a 20-cm. SEC column with a buffer containing 1*M* DMF at pH 4.2 is shown in Fig. 2. The resolution was better than

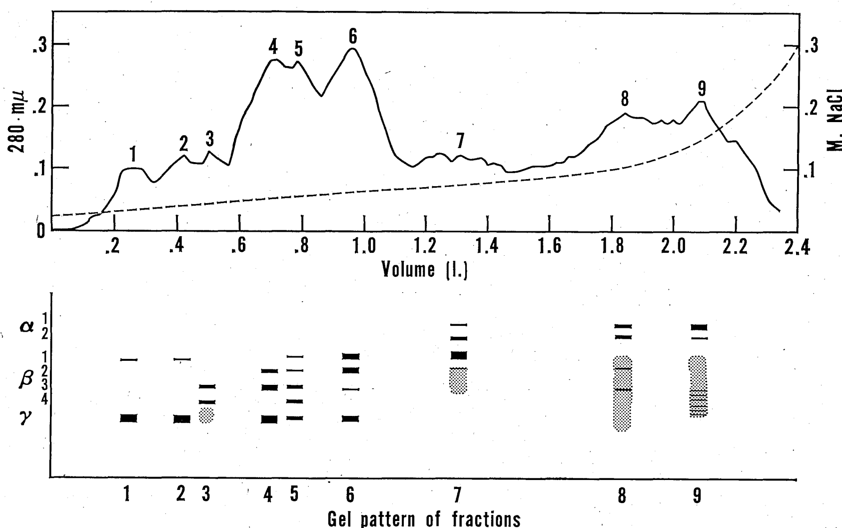

Fig. 2. Chromatographic separation of gliadin on sulfoethyl cellulose (SEC) 4.0 × 20-cm. column. Buffer: 1*M* DMF, 0.005*M* sodium acetate, 0.05*N* acetic acid, pH 4.2. Broken line shows salt gradient in molarity. Starch-gel electrophoretic patterns of fractions illustrated in lower portion.

that obtained with CMC. On a PAC column with the same buffer the separation was not as good, so all further studies were carried out on SEC.

To determine the most effective pH for operation of SEC columns, they were tested with buffers of pH values from 4.5 to 1.4. A separation at pH 1.4 is shown in Fig. 3. At this low pH, resolution improved

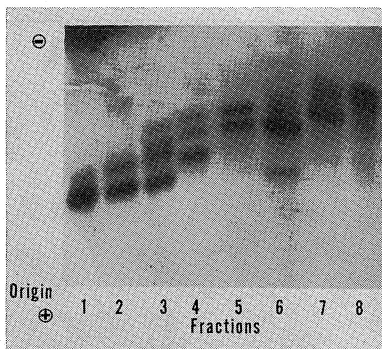

Fig. 3. Starch-gel electrophoretic patterns of chromatographic fractions of gliadin on SEC, 4.0 × 20-cm. column. Buffer: 2*M* DMF, 0.1N acetic acid, 0.03*N* HCl, pH 1.5.

with less trailing into the next peak and fewer components in some peaks; also, the order of elution was more consistent with their electrophoretic mobility. Separation between the alpha and beta components is well delineated. The low pH, however, introduced the possibility of peptide and amide hydrolysis in the protein before solvent removal.

To determine the optimum column height, various lengths as given under "Materials and Methods" were tested for the best separation of gliadin. A 100-cm. column was tried, but flow rate was too slow. As shown in Fig. 4, an improvement in resolution was achieved with a

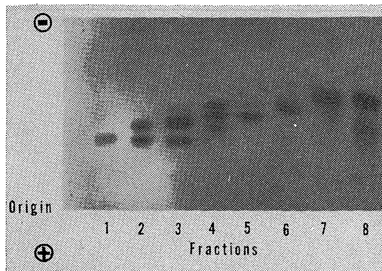

Fig. 4. Starch-gel electrophoretic patterns of chromatographic fractions of gliadin on SEC, 2.8 × 50-cm. column. Buffer: 2*M* DMF, 0.05*N* acetic acid, 0.02*N* HCl, pH 1.9.

50-cm. column at pH 1.9 as compared to a 20-cm. column at the same or even lower pH. By making the column excessively long, diffusion caused some spreading of the peaks, but this was not a problem with the 50-cm. column.

Fractions isolated from the columns were rechromatographed to determine whether additional resolution was possible and whether the positions of elution were reproducible. Rechromatography of certain of the peaks under the same conditions generally gave a single peak consisting of the same starch-gel electrophoretic components. Figure 5

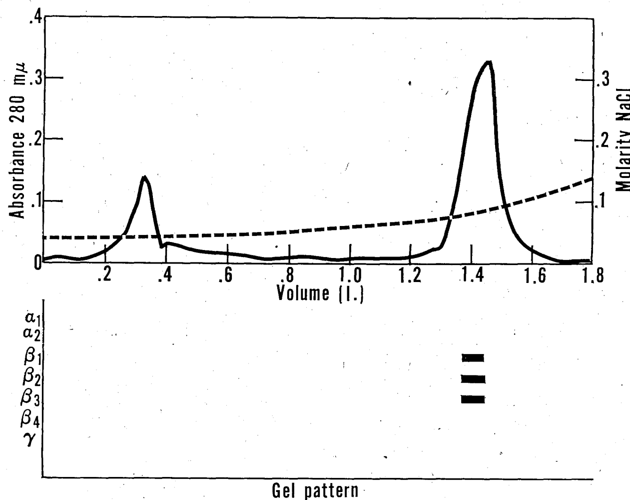

Fig. 5. Rechromatography of a gliadin fraction on SEC, 2.8 × 50-cm. column. Buffer: 2*M* DMF, 0.05*N* acetic acid, 0.015*N* HCl, pH 2.1. Fraction 4 from Fig. 4. Broken line shows salt gradient in molarity. Starch-gel electrophoretic pattern illustrated in lower portion.

illustrates the results of rechromatographing peak 4 shown in Fig. 4 on a similar column. On both occasions the β_1, β_2, and β_3 gliadin components appear in a single peak. The first small peak obtained during rechromatography was devoid of protein and may be a pigment artifact.

Proteins having mobilities identical to the β_1, β_2, and β_3 gliadins are present in other peaks eluted from the column shown in Fig. 4. Two possibilities may account for this unique behavior: 1) tightly bound intermolecular complexes may occur between gliadin proteins under the conditions of chromatography, and 2) there may be more than one component having a similar electrophoretic mobility in starch gel made with aluminum lactate-3*M* urea buffer.

A preparation of gamma-gliadin obtained by continuous-flow paper electrophoresis (7) was applied to a 50-cm. SEC column with $2M$ DMF used in the buffer at pH 2.1. Three different peaks were obtained (Fig. 6), each of which had a starch-gel electrophoretic mobility identi-

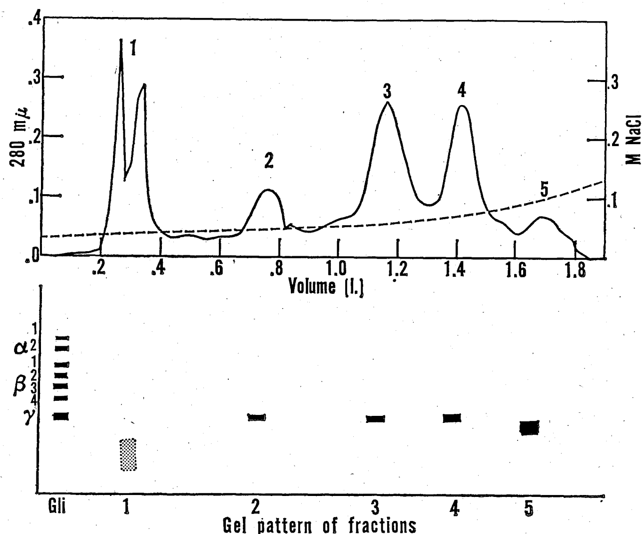

Fig. 6. Chromatographic separation of gamma-gliadin on SEC, 2.8 × 50-cm. column. Buffer: $2M$ DMF, $0.05N$ acetic acid, $0.015N$ HCl, pH 2.1. Broken line shows salt gradient in molarity. Starch-gel electrophoretic pattern of fractions illustrated in lower portion.

cal to that ascribed to gamma-gliadin. The appearance of three peaks may arise from the existence of three different gamma-gliadin components, or from aggregation taking place on the column with formation of monomer, dimer, and tetramer, each having distinctly different elution volumes. The presence of urea and low ionic strength in the starch gel may dissociate aggregates to yield electrophoretically identical material in the starch-gel patterns. Similar aggregation of beta components would account for the multiplicity of peaks containing various beta constituents.

To eliminate the possible interference with separation caused by aggregation, increased levels of solutes known to dissociate gliadin proteins were added to the elution buffer. Initially the concentration of DMF used was $2M$, with only small changes occurring in the elution patterns. Urea was then tested at various levels from 1 to $8M$. At low pH, urea solutions of high concentration were unstable and yielded ammonia, which raised the pH and the ionic strength. Therefore, experiments with urea were conducted at pH values above 3.0.

Solutions containing 1 and 3*M* urea gave results similar to 1 and 2*M* DMF. Using 6*M* urea caused the gamma component to come off in one large peak having three slight shoulders. Only the last third of the peak contained another gliadin component, β_3. The order of elution was the same as the electrophoretic mobility on a starch gel. Only one peak contained more than two components, except for the alpha fractions which contained some higher-MW impurities.

Using 8*M* urea in the buffer gave a large initial peak that consists almost exclusively of gamma-gliadin (Fig. 7). Peak 5 was fairly pure

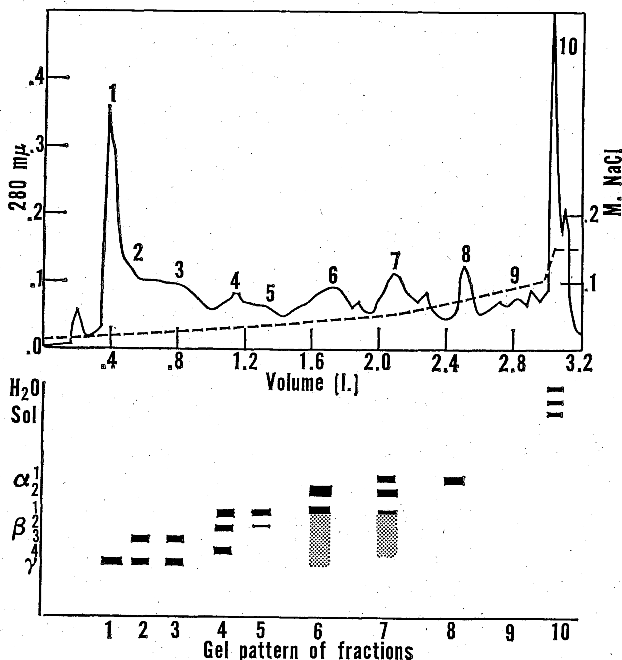

Fig. 7. Chromatographic separation of gliadin on SEC including starch-gel electrophoretic pattern of fractions. Buffer: 8*M* urea, 0.03*N* acetic acid, 0.01*N* HCl. Broken line shows salt gradient in molarity. Dotted area in fractions 6 and 7 denotes streaking in gel pattern.

β_1, whereas 8 was exclusively α_1 gliadin. Fraction 4 still consisted of three components, but this may be caused by overlapping of adjacent peaks. Though this last procedure resulted in purification of part of the gamma and α_1 components and yielded nearly pure β_1, there are some problems involving the use of 8*M* urea. Except for the first and last peaks, which were quite sharp because of rapid change in ionic strength, most peaks were broad. Also, the concentrated urea solution

compacted the column, thereby decreasing flow rate; in addition it solubilized some of the cellulose, which precipitated again when the urea concentration was lowered during dialysis. Furthermore, extensive dialysis and larger volumes of distilled water are required to remove excess urea.

Discussion

Several innovations in gliadin chromatography have been introduced in the present study. For example, SEC increased the capacity of the columns, facilitated use of lower pH, and gave better separation of gliadin proteins than CMC. The Varigrad device allowed more discrete separations to be obtained by adjusting the gradient to conform to optimum resolution. A very gradual increase in the ionic-strength gradient was necessary to get the best separation of the gamma and beta gliadins. Once the gradient system with the Varigrad was established for a given column, the elution pattern of proteins was highly reproducible, and certain proteins were eluted when the solution reached definite ionic strengths. In general, on a specific column and elution system, higher concentration of solutes such as urea or DMF reduced the ionic strength necessary to remove a given protein.

The use of 1*M* DMF in the method of Simmonds and Winzor (4) evidently prevented precipitation of gliadin proteins but possibly not aggregation. Winzor (14) demonstrated that an individual protein fraction of gluten does undergo aggregation in 1*M* DMF, 0.09*M* NaCl-0.01*M* sodium acetate at pH 4.1. The aggregation induced by ionic-strength increase during chromatography could possibly result in resolution of molecules of different degrees of molecular association, in accordance with the Gilbert (15) principle.

The function of high concentrations of urea in improving chromatographic separation of various proteins on ion-exchange cellulose has generally been attributed to reduction of absorption by the protein to the cellulose (16). Possibly urea affects disaggregation of the protein to give a uniform chromatographic behavior. Molecular aggregation of insulin and casein proteins under the conditions for chromatography has been discussed in the literature (16). The improved elution of gliadin components from more highly acidic columns may also result, in part, from dissociation of proteins by electrostatic repulsion at low pH. The change in elution patterns with higher concentrations of urea or with buffers of lower pH indicates that in many of the systems tested aggregation phenomena may be responsible for the reappearance of similar electrophoretic gliadin proteins in different peaks.

The application of SEC columns with buffers containing high con-

centrations of urea should permit isolation of individual proteins, such as the γ, β_1, and α_1, for detailed chemical and physical studies. The alpha fractions containing the higher-MW materials can be further purified by passing them over a Sephadex G-75 or G-100 column. Rechromatography with a different buffer may also help in further separating some of the beta components. Possibly it will still be necessary to supplement column chromatography with other tools, such as electrophoresis or gel filtration, to effect complete separation of each protein.

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