One- and Two-Dimensional (Two-pH) Polyacrylamide Gel Electrophoresis in a Single Gel: Separation of Wheat Proteins

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ABSTRACT

A procedure is described for one- and two-dimensional polyacrylamide gel electrophoresis of wheat proteins with commercially available equipment. The first separation is carried out at pH 3.1 and the second at pH 9.2. Both separations are carried out in the same gel, leading to enhanced resolution. The system should also be applicable to other types of proteins and could be modified to incorporate alternate buffer systems.

Key words: Albumins, Gliadins, Globulins, Wheat histones

Two-dimensional (2-D) electrophoresis has been extensively used for separation and characterization of proteins. Commonly, the first-dimension separation occurs in a gel cylinder or strip, which is then positioned across the top of a second slab gel for separation in the second dimension. In this way, nearly any two one-dimensional (1-D) electrophoretic techniques can be combined to improve resolution of a protein mixture.

Mecham et al (1978) introduced a 2-D electrophoresis method in which both separations occur in one polyacrylamide gel slab; the 1-D separation was carried out at pH 3.2 (aluminum lactate buffer), and the second at pH 9.2 (Tris-glycine buffer). Kaltschmidt and Wittmann (1970) used a 2-D, two-pH method to separate ribosomal proteins, but their procedure involved insertion of the 1-D gel into the second gel. Mecham et al (1978) and D. K. Mecham (*unpublished*) found that carrying out 2-D electrophoresis in a single gel greatly reduced distortion of protein bands that occurred as the proteins cross the interface between the first and second gels, which resulted in improved resolution when compared with a two-gel approach. We think it likely that similar distortion of bands as they move through the interface between gels is responsible for the flattening of bands commonly seen in 2-D procedures (see O'Farrell 1975, for example). Thus, bands vertically oriented in the 1-D gel (as positioned at the top of the second-dimension gel slab) frequently become horizontally oriented during the second-dimension separation, so that the resulting spots appear somewhat flattened.

Gliadins are the major storage protein fraction of wheat endosperm; they constitute a complex mixture of similar components, all rich in glutamine (30–55 mol %) and proline (15–30 mol %) and having relatively few ionizable side chains. Their electrophoretic patterns are extensively used for identifying wheat varieties (Wrigley et al 1982), and in genetic and biochemical investigations of the complex loci coding for these proteins (Mecham et al 1978, Shewry et al 1984).

In the procedure of Mecham et al (1978) more than 24 hr was required to complete both dimensions, and the 6-mm thick gels required another 24–48 hr of staining before patterns could be viewed. In addition, modifications to commercial apparatus were required to carry out the analysis. In this paper we report a modification of the procedure of Mecham et al (1978) that is useful for separating wheat gliadins and other wheat endosperm proteins (albumins and globulins). The first dimension is based on the procedure of Laurière and Mossé (1982) with some modifications. Our modified procedure requires only commercially available equipment, uses thin (1.5 mm) gels, can be completed so that a stained pattern can be viewed in about 8 hr, and optimum staining requires only a few more hours.

MATERIALS AND METHODS

Reagents

Acrylamide and *N,N'*-methylenebisacrylamide (white label) were from Eastman Kodak Co. Aluminum lactate was from Riedel-de Haen (F. R. G.), obtained through the U.S. distributor, Crescent Chemical Co. Hydrogen peroxide was 30% AR from Mallinckrodt Chemical Co. All other chemicals were reagent grade. Water was distilled (metal still) and then deionized by passage through a mixed-bed ion-exchanger.

Histones

A purified wheat histone preparation was a gift from R. Quatrano and S. Spiker, Oregon State University, Corvallis (Spiker 1982).

Endosperm Protein Extraction

Gliadins were extracted by the method of Mecham et al (1978) with minor modifications. Single seeds were extracted by grinding them with 1.5*M* dimethylformamide (DMF) in a mortar (0.01 ml per mg of seed) for 3–5 min. The slurry was transferred with a pipet to a 2.5-ml plastic centrifuge tube and centrifuged ($\sim 10{,}000 \times g$) for 10 min with a table top centrifuge (model 235, Fisher Scientific Co., Pittsburgh, PA). Alternatively, to obtain more concentrated solutions, seeds were crushed in a mortar, transferred to the centrifuge tube, DMF extractant added, and the mixture allowed to stand for about 1 hr with occasional mixing (vortex mixer). Albumins, or albumins and globulins, were extracted with water or 4.25 m*M* aluminum lactate buffer (pH 3.1) at a solvent to seed ratio of 3:1 (0.003 ml/mg). All extracts were made 20% in sucrose before electrophoresis to increase their density for sample application to wells under the top electrode buffer. The sucrose included a small amount (10 mg/30 g sucrose) of tracking dye (methyl violet for gliadins and methyl green for albumins and globulins) that provided a measure of the extent of protein migration. These dyes migrate slightly faster than the respective groups of proteins during first-dimension electrophoresis.

Solutions for Gel Preparation and Electrophoresis

The following solutions were prepared: A) Acrylamide, 28.0 g; *N,N'*-methylenebisacrylamide, 1.2 g; and H_2O added to 100 ml. B) Potassium hydroxide, 3.5 g; lactic acid (85%), 25 ml; and H_2O added to 100 ml. C) Aluminum lactate, 6.25 g; lactic acid (85%), 10 ml; and H_2O added to 100 ml. D) Silver nitrate, 17 mg/ml H_2O. E) Ammonium persulfate, 90 mg/100 ml H_2O. F) Hydrogen peroxide, 1% (100 μl of 30% H_2O_2 diluted to 3.0 ml with H_2O). G) Solution A, 17 ml; solution B, 2 ml; ascorbic acid, 20 mg; ferrous sulfate, 2.5 mg; and H_2O added to 100 ml. Solutions A, B, and C were filtered through Whatman No. 5 filter paper immediately after they were prepared; these solutions can be stored at 4°C for a few weeks. Solution G was divided into 10-ml portions, which were stored in

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stoppered vials at −10°C. Solutions D, E, and F were freshly prepared for each use. Ammonium persulfate was stored over desiccant to prevent absorption of water. Hydrogen peroxide (30%) was stored at room temperature.

Gel Casting and First-Dimension Electrophoresis

The first-dimension separation was carried out with a Protean dual 16-cm slab cell vertical electrophoresis apparatus (Bio-Rad, Richmond, CA) at pH 3.1. Before casting polyacrylamide gels, glass plates were thoroughly cleaned with dichromate-sulfuric acid solution for 24 hr (although less time may be sufficient), rinsed thoroughly with water for about 5 min, soaked for 5 min in a 0.2% solution of Kodak (U.S.A.) Photoflow-200, and thoroughly air-dried. A detergent-based cleaning solution (Cleaning Concentrate, Bio-Rad; diluted to 4% with water) can also be used to clean glass plates. It is important that glass plates be thoroughly clean and scratch-free, or gels may be difficult to remove from the plates following electrophoresis. We therefore avoid cleaning the plates with brushes, and use only soft sponges on them.

For casting two (separating) gels, the following solution was prepared to produce a 7% (w/v) gel with 0.3% (w/v) crosslinking: 20 ml of solution A, 1.6 ml of solution B, 0.8 ml of solution D, 40 ml of solution E, and water added to 80 ml. The final pH of this solution is 3.1–3.2; no adjustment is necessary. The solution was deaerated for 2 min in a suction flask attached to a water aspirator (longer deaeration will result in too rapid polymerization) and poured between the glass plates (16 × 18 cm with 1.5-mm spacers) to within 2-3 cm of the top; water-saturated butanol was then layered gently on the solution surface to exclude air and provide a smooth surface. While the gels were polymerizing, 5 L of electrode buffer was prepared by 50-fold dilution of solution B. Gels usually polymerized sufficiently in 20 min, but sometimes required up to 1 hr for a reasonably firm gel to be formed. Polymerization can be recognized by the appearance of a second phase interface just below the surface of the butanol/aqueous solution interface. After polymerization, the butanol and any unpolymerized material at the top of the gel were carefully removed with a syringe and metal needle, and the top of the gel was rinsed several times with electrode buffer. Subsequently, gels were prerun for 1 hr at 45 mA per gel with 4.5 liters of electrode buffer in the bottom chamber and 0.5 liters in the upper chamber. Tap water (water temperature usually about 20°C year-round at this location) was run through the cooling coils of the apparatus to diminish gel heating during a run.

After the prerun, the top buffer was discarded and the top (stacking) gel was prepared. Ten milliliters of solution G was taken from the freezer and thawed. When thawing was just complete, 15 μl of deaerated solution F (H_2O_2) was added to solution G, and the cold solution poured onto the separating gel; the combs were quickly inserted because polymerization is rapid. Alternatively, before the top gel is poured, combs can be loosely positioned between the plates at a slight angle to allow the stacking gel solution to be applied with a 10-ml pipet; combs can then be quickly adjusted to their proper position; this entire procedure can be accomplished within about 30 sec of adding hydrogen peroxide to solution G.

After 5–10 min, combs were removed with a very gentle rocking motion to avoid damaging the slots. The slots were rinsed free of any remaining polyacrylamide solution or polymerized debris with aluminum lactate buffer, pH 3.1 (solution C, diluted 50-fold and adjusted to pH 3.1 with lactic acid). The top reservoir was then reassembled, and the top of the gel and reservoir filled with 0.5 L of aluminum lactate buffer (solution C, diluted 50-fold and adjusted to pH 3.1 as above). Protein extracts (7–25 μl) were then loaded into slots (a single slot is loaded when a 2-D analysis is to be carried out), and gels were run at 45 mA each (90 mA for two gels run simultaneously) until the appropriate tracking dye reached the bottom of the gel. Voltage was approximately 500 V. After completion of the run (about 2 hr for gliadins), gels were removed by gently prying the glass plates apart with a plastic spacer (from casting apparatus); gels usually adhere to one or the other of the plates. Then, while holding the plate with gel attached (gel side down) horizontally over a tray, we pried a corner of the gel away from the plate with the spacer; the gel then usually peels away from the plate under its own weight. Occasionally, gels do not peel satisfactorily from the second plate; immersing the plate and gel in the staining solution of second-dimension buffer (gel side up) usually leads to satisfactory recovery of an intact gel.

Second-Dimension Electrophoresis

When a 2-D separation was to be carried out, a single sample was loaded into a central slot (or a slot displaced slightly from the center, depending on the nature of the 2-D pattern) for first-dimension electrophoresis. Somewhat larger samples (15–25 μl) were usually loaded when 2-D electrophoresis was to be carried out. Sometimes the same sample was also loaded in an end slot: this gel pattern was to be cut off and stained to provide a 1-D reference pattern before the second-dimension separation was carried out.

After the first electrophoretic separation, gels were removed from the plates and equilibrated for 30–45 min with 1 L of 0.125*M* tris(hydroxymethyl)aminomethane, 0.025*M* glycine, 1.5*M* DMF, pH 9.2. (DMF is important to maintain solubility of wheat gliadin proteins, but may be excluded for proteins readily soluble at pH 9.2; even so, the DMF did not result in significant diffusion or loss of protein bands in the gel.) During equilibration, gels were supported by a stainless steel screen raised slightly above the bottom of a glass tray of buffer, so that the buffer could be stirred with a magnetic stirring bar. Alternatively, trays may be shaken gently to achieve equilibration.

The second-dimension electrophoresis was carried out with a Pharmacia horizontal electrophoresis apparatus (model FBE 3000). A transparent cellulose acetate sheet was placed on the single cooling plate of the apparatus and the gel positioned on this sheet so that the electric field would be applied at 90° to the field of the first dimension. Cellulosic sponges (2.5 mm thick), cut to proper size, were saturated with equilibration buffer in the buffer chambers and then laid over the edges of the gel to connect buffer and gel. (Our source of sponges is no longer in business, but sponges from Spontex Distributors, Long Island City, NY may be suitable.) The gel surface may be protected with a thin plastic film (Saran wrap or equivalent), or another cellulose acetate sheet may be placed on top of the sponges before the lid of the apparatus is put in place to prevent dehydration of the gel surface. Both methods seemed satisfactory to us. Electrophoresis in the second dimension was then carried out for 4 hr at 20 mA; this time may need to be decreased, should there be evidence that proteins have run off the end of the gel, or increased if the pattern appears too compressed.

Gel Staining and Drying

After 1- or 2-D separations, the gels were stained with a solution of 0.02% Coomassie Brilliant Blue R250 in 5% ethanol, 12% or 6% trichloroacetic acid (TCA) (the dye was dissolved first in 95% ethanol, filtered, and then added to a TCA solution). Gels were stained overnight, but patterns could be seen in about 30 min for 1-D separations. Separations in the pH 9.2 second-dimension buffer were visible only after several hours, and 8–16 hr were required for complete development of the pattern. Destaining is not required before photography but can reduce background stain. Gels are rinsed in water containing a few drops of a nonionic detergent (such as Triton X-100) in the rinse water to remove incidental surface stains before photography. Gels were photographed with a Polaroid model MP-3 camera (Polaroid type 52 or 55 black and white film, Wratten G-15 [yellow-orange] filter).

After being photographed, gels may be dried for preservation. A gel is placed on a cellulose acetate sheet and then on a glass plate. The upper gel surface is covered with a porous cellophane membrane (Bio-Rad 1650922) and the edges clamped to stretch the membrane tightly and smoothly over the gel surface. The membrane and gel then air dry (usually in a fume hood because of rapid air flow) for 24–48 hr. The dry gel and the cellophane membrane, which remains attached, are then removed from the cellulose acetate bottom sheet. Gels dried in this manner can be retained indefinitely for future comparisons and may also exhibit enhanced color of faint spots.

RESULTS AND DISCUSSION

1-D Method

We found polymerization with the silver nitrate system of Laurière and Mossé (1982) too slow and had difficulties in obtaining good polymerization around the comb, so we modified their single-gel system into a two-gel system in which the running gel is polymerized with silver nitrate and ammonium persulfate (Narayan et al 1965), and a top gel polymerized with hydrogen peroxide (Tkachuk and Metlish 1980) is then added. This top gel may contribute a partial stacking effect, but some gliadin components, particularly the larger ω-gliadins, begin to separate in the top gel; this may result from a sieving effect.

A 1-D separation of gliadins from ten different bread and durum (pasta) wheat varieties using this type of gel is shown in Figure 1. Bands with greater mobilities than α-gliadins are apparently albumins or globulins that are extracted from the seed by the DMF-water extractant (D. D. Kasarda and N. Fulrath, *unpublished*). Aluminum or sodium lactate buffers at pH 3.1 or 3.2 are commonly used for gliadin electrophoresis (see Kasarda et al 1976, or Wrigley et al 1982 for reviews); our modification of the Laurière and Mossé (1982) procedure appears to provide resolution similar to or better than that of other recently described approaches (Laurière and Mossé 1982, Bushuk and Zillman 1978, du Cros and Wrigley 1979, Khan et al 1983, Lookhart et al 1982, Gaponenko et al 1983). Gaponenko et al (1983) attribute the sharpness of bands in their system to use of a 10.5% polyacrylamide gel rather than the 6-7% gel concentrations most commonly used. We therefore compared 7, 10, and 15% gels in our method (results not shown). Although some bands were slightly sharper, no marked improvement in resolution was observed using higher gel concentrations, and longer electrophoresis times were required.

In order to test our method with a class of proteins that has rather different characteristics from the gliadins, we arbitrarily chose the wheat histones. A 1-D separation of the wheat histone mixture is shown in Figure 2. The separation achieved is as good or better than similar acid separations of histones (Spiker 1982, Chiu and Irvin 1980).

2-D Method

Figure 3 shows a 2-D separation of gliadins from the wheat variety Chinese Spring. Comparison of this pattern with track I of Figure 1 demonstrates the power of our 2-D system to resolve components that overlap in a 1-D separation. Mobilities in the second dimension also provide information useful in differentiating components on the basis of their differences in ionizable amino acids.

Separated gliadin components retain the narrow band shape of the first dimension throughout the second-dimension separation (Fig. 3), mainly because the separation was performed in a single gel. We suspect that other 2-D methods would also show diminished horizontal band spreading in the second dimension if carried out in one rather than two gels.

Resolution of gliadins with our 2-D method, in terms of total number of components separated, is similar to that achieved by Wrigley and Shepherd (1973) and du Cros et al (1983), who used higher resolution isoelectric focusing in the first dimension and two separate gels. Since carrying out 2-D electrophoresis in one gel should enhance resolution, the combination in a single gel of isoelectric focusing in the first dimension with electrophoresis in the second dimension might provide even better resolution.

Our pattern of Chinese Spring gliadins (Fig. 3) is moderately similar in the grouping of components to that of Wrigley and Shepherd (1973). This similarity results partly because both approaches use lactate electrophoresis as one dimension but may also indicate that electrophoresis of gliadins at pH 9.2, as in our second dimension, separates gliadins approximately according to isoelectric points (Lafiandra et al 1984).

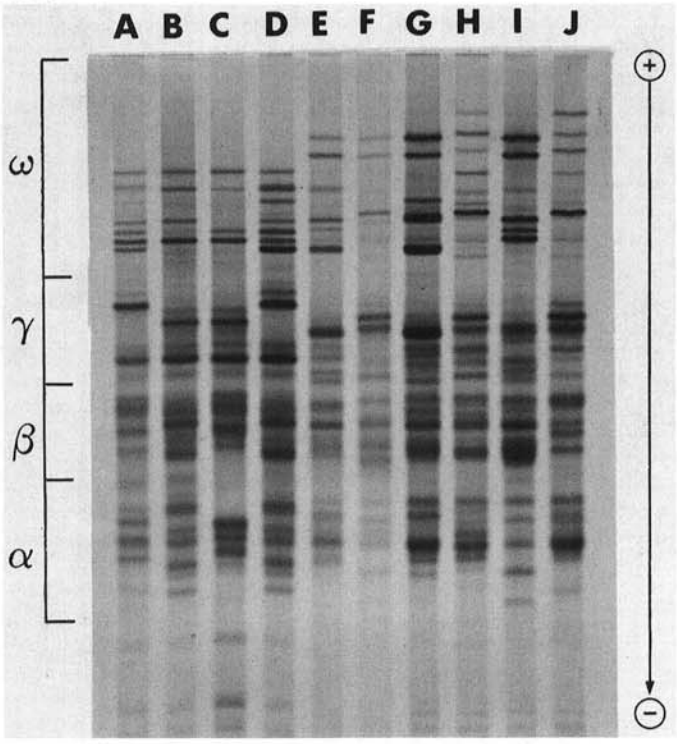

Fig. 1. One-dimensional electrophoresis of gliadin proteins from durum (tetraploid) (A-D) and bread (hexaploid) wheat varieties (E-J). Aluminum lactate buffer, pH 3.1 (see text). Migration was from top to bottom (+ to −). **A,** Lakota; **B,** Cappelli; **C,** Agathe; **D,** Produra; **E,** INIA 66R; **F,** Anza; **G,** Wichita; **H,** Scout 66; **I,** Chinese Spring; **J,** Cheyenne. Greek letters refer to usual electrophoretic mobility designations of gliadin groups (Mecham et al 1978, Kasarda et al 1976).

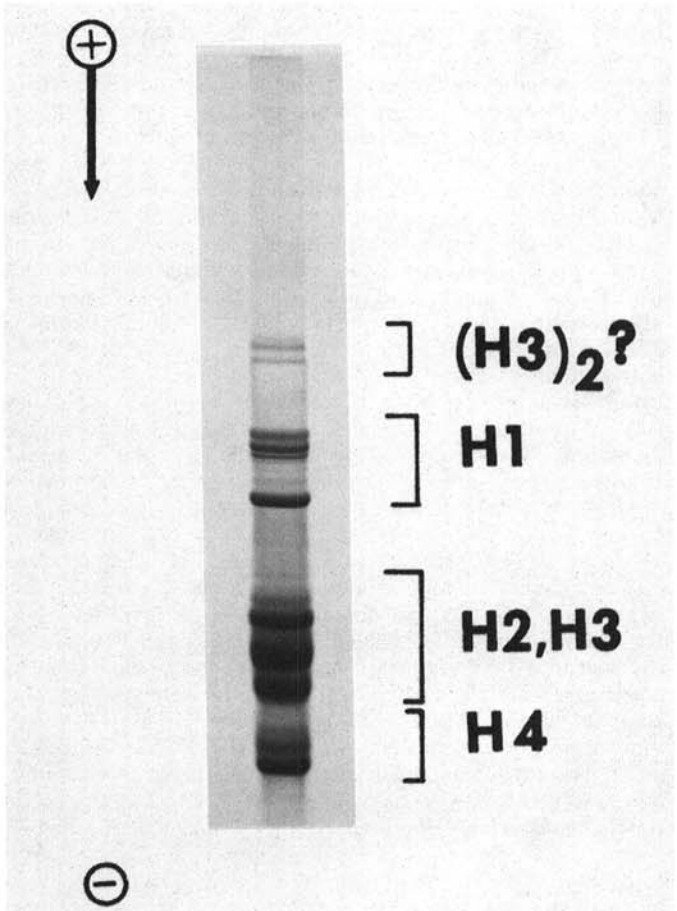

Fig. 2. One-dimensional electrophoresis of wheat histone proteins. The designations of the wheat histones are tentative and based on those indicated by Spiker (1982) and Hurley (1977) for wheat and maize histones.

In Figure 4, we compare the patterns of Wichita gliadins obtained by the method of Mecham et al (1978) (Fig. 4A) and by the present method (Fig. 4B). Although patterns are similar, the present method gives less distortion of bands and sharper patterns; in addition, it requires one-third the time and one-third as much sample as the procedure of Mecham et al (1978). These improvements result mainly from the use of relatively thin, 1.5-mm gels rather than the 6-mm gels that were required in the procedure of Mecham et al (1978). The thicker gels required longer equilibration, electrophoresis, and staining and destaining times—all of which contribute to band broadening through diffusion in the gels. The use of the top (stacking) gel in our procedure may also contribute to improved resolution in our present procedure.

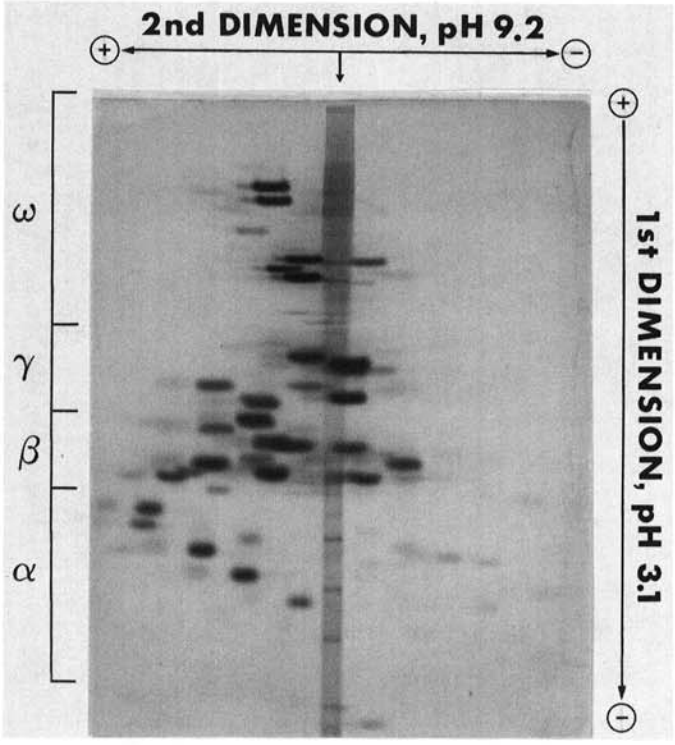

Fig. 3. Two-dimensional (2-pH) electrophoretic separation of gliadins from the wheat variety Chinese Spring. Origin of first dimension (lactate buffer, pH 3.1) was at top, center. Conditions for first dimension and component designations are as in Figure 1. In the second dimension (Tris-glycine, DMF, pH 9.2), the electric field was applied at right angles to that of the first dimension. See text for details.

Our 2-D procedure is also useful for separation of albumin and globulin proteins. In Figure 5, we show 2-D separations of Chinese Spring proteins extracted with water (Fig. 5A) and with aluminum lactate buffer, pH 3.1 (Fig. 5B). Most water-soluble proteins of wheat endosperm migrate more rapidly than gliadins in the lactate buffer system (pH 3.1). When electrophoresis time is chosen to optimize gliadin separations, most of the water-soluble (and salt soluble) proteins migrate off the gel (Figs. 1, 3, and 4); to prevent this from occurring and keep these proteins on the gel, the electrophoresis time for albumins and globulins must be reduced to less than half that used for the gliadins.

The acidic lactate buffer (Fig. 5B) is much more effective in extracting gliadins than is water (Fig. 5A)—as evidenced by the heavy staining of the top third of the gel of Figure 5B. Other considerable differences in protein composition are also evident between these extracts. Some components were extracted by either solvent (based solely on similarity of electrophoretic mobility), whereas others were unique to one or the other. Some components did not migrate in the second dimension (see the bottom third of Fig. 5A) because they are either isoelectric at pH 9.2 or are insoluble in our pH 9.2 buffer. These results demonstrate that important wheat albumin and globulin proteins, such as inhibitors of mammalian and insect α-amylases (Kasarda et al 1976, Buonocore et al 1977, Garcia-Olmedo et al 1982), may be successfully analyzed by our procedure.

We attempted to separate wheat histones by our 2-D method but found that the 2-D pattern was the same as that obtained in one dimension (Fig. 2). Failure of histones to migrate in the second dimension might result from insolubility in the second-dimension

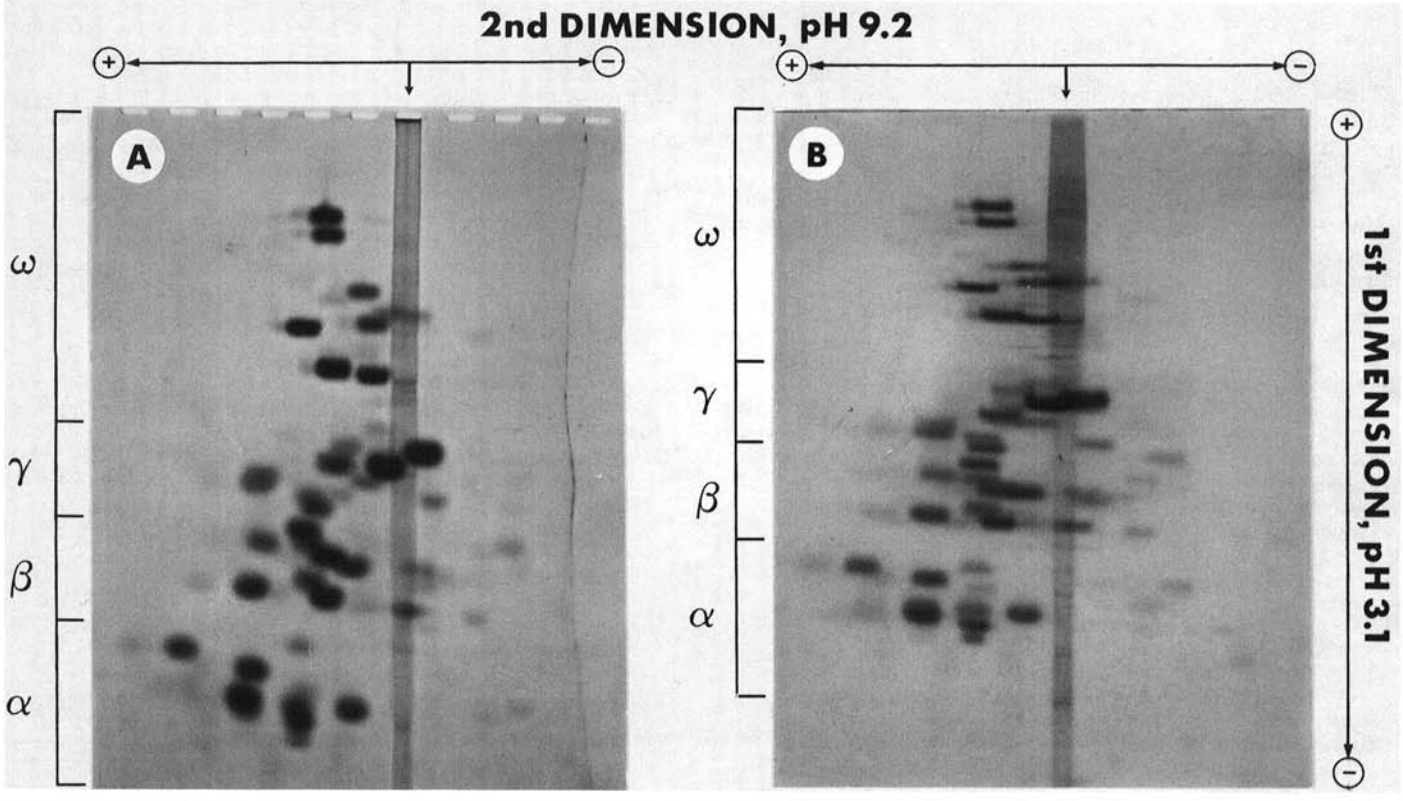

Fig. 4. Two-dimensional (2-pH) electrophoresis of Wichita gliadins by the method of Mecham et al (1978) (**A**) and that described in this paper (**B**). Conditions and band designations are as in Figures 1 and 2.

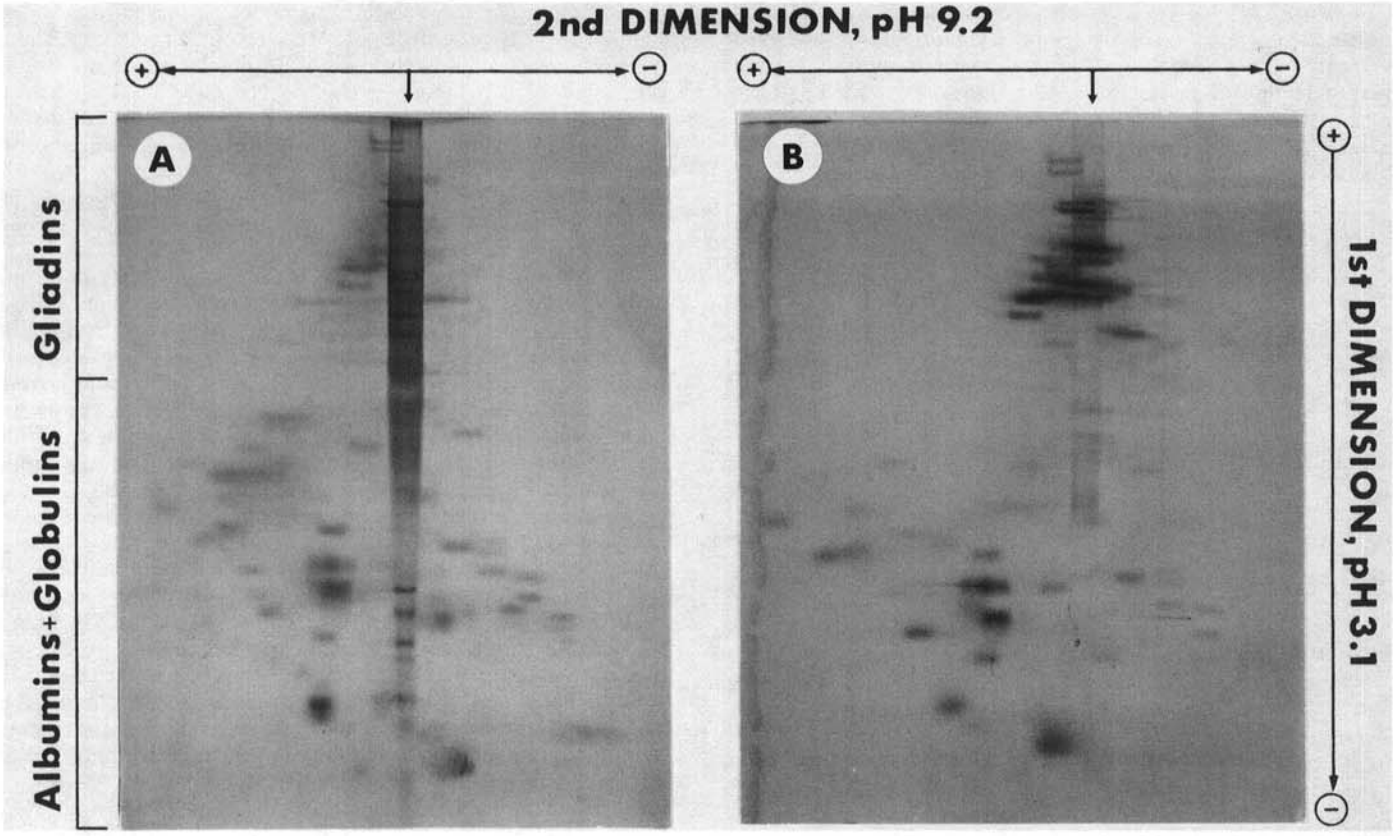

Fig. 5. Two-dimensional (2-pH) electrophoretic patterns of wheat albumins and globulins (and gliadins) extracted from the wheat variety Chinese Spring by **(A)** water and **(B)** 4.25 m*M* aluminum lactate buffer (pH 3.1).

buffer, or from their high content of basic amino acids causing them to be isoelectric at pH 9.2 (Spiker 1982). We suspect, however, that such problems will be rarely encountered with most other proteins.

CONCLUSIONS

The 1- and 2-D electrophoretic methods we describe provide rapid and efficient resolution of wheat gliadins, albumins, and globulins. Our procedure overcomes the gel fragility we have noted using hydrogen peroxide as the polymerization catalyst, making it easier to carry out 2-D separations on a single gel. Our procedure should also be readily adaptable to other commercially available apparatus and combinations of buffers, making it generally useful for proteins other than gliadins.

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