Two-Dimensional Electrophoretic Analysis of Wheat Kernel Proteins[1]

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ABSTRACT

Wheat (*Triticum aestivum*) kernel proteins were fractionated by extraction or solubility in water, 0.5*M* NaCl, 70% ethanol, or 1% sodium dodecyl sulfate (SDS)/2% 2-mercaptoethanol. Protein components of each fraction were analyzed by two-dimensional electrophoresis. Both isoelectric focusing and nonequilibrium pH gradient electrophoresis were employed in the first dimension, and SDS slab gel electrophoresis was used in the second dimension. Albumin and globulin fractions were found to have very different electrophoretic patterns, each with its own unique characteristics. The alcohol-extracted proteins and the SDS-extracted proteins had rather similar electrophoretic patterns. About 300 spots were resolved in the water- or salt-extracted proteins, and about 180 spots in the water-and-salt-nonextracted proteins. The nomenclature of wheat kernel proteins is discussed in light of our results.

Key words: Gliadin fraction, Glutenin fraction

Historically, cereal proteins have been classified by their solubility characteristics. Osborne (1970) defined four sets of wheat kernel proteins as: albumins (soluble in water), globulins (soluble in neutral saline), gliadins (soluble in 70–90% alcohol), and glutenins (soluble in dilute acid or alkali). Albumin and globulin fractions constitute about 12 and 5% of total wheat proteins, respectively (Chen and Bushuk 1970a). They are found mainly in the embryo and aleurone layer of the wheat kernel (Payne et al 1982). Gliadin and glutenin fractions are the major storage proteins of wheat endosperm. Each accounts for about 40% of total protein in the wheat kernels (Khan and Bushuk 1979). Gliadin and glutenin fractions are said to impart the viscosity and elasticity, respectively, to the viscoelastic properties of gluten (Khan and Bushuk 1979).

In studies of wheat proteins, the Osborne solubility criteria and nomenclature have been used a great deal, although each solubility fraction of wheat kernel proteins has been shown to be quite heterogeneous by chromatography and electrophoresis (Woychik et al 1961; Kelley and Koenig 1962; Feillet and Nimmo 1970; Chen and Bushuk 1970b,c; Bietz and Wall 1972; Petrucci et al 1974; Bietz et al 1977; Khan and Bushuk 1978; Payne and Corfield 1979; Jones et al 1982). At least as serious a limitation in the Osborne nomenclature is an apparently substantial overlap in the sets of proteins in the gliadin and glutenin fractions (Bietz and Wall 1973, 1975; Danno et al 1974; Payne and Corfield 1979; Field et al 1982; Jackson et al 1983).

The two-dimensional gel electrophoresis techniques developed by O'Farrell (1975) and O'Farrell et al (1977) are useful for analyzing complex mixtures of proteins. Originally, the system employed isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulfate (SDS) slab gel electrophoresis in the second dimension (O'Farrell 1975). This approach was especially good for analysis of neutral and acidic polypeptides. Later, O'Farrell et al (1977) combined nonequilibrium pH gradient electrophoresis (NEPHGE) and SDS slab gel electrophoresis to separate basic polypeptides. Two-dimensional gel electrophoresis of both types (IEF/SDS and NEPHGE/SDS) has been used to study the proteins of wheat endosperm and the genetic control of those proteins (Brown et al 1979, 1981; Brown and Flavell 1981; Holt et al 1981; Jackson et al 1983).

In this study, we employed the O'Farrell two-dimensional gel electrophoresis techniques to analyze all four Osborne solubility fractions of wheat kernels (i.e., the albumin, globulin, gliadin, and glutenin fractions). The purpose of this study was to analyze the complexity of each fraction in detail, to compare the fractions to each other, and to reexamine the classification of wheat proteins by their solubilities.

MATERIALS AND METHODS

Sample Preparation

Kernels of a common wheat cultivar, Newton, grown in Kansas in 1982, were used in this study. Newton is a hard red winter hexaploid wheat with a high breadmaking quality. Newton wheat kernels were generously provided by R. C. Hoseney, Department of Grain Science, Kansas State University. Thirty kernels of wheat (about 1 g) were used for an extraction. The kernels were finely ground with mortar and pestle.

The albumin and globulin fractions (together) were extracted twice with 3 ml of 0.5*M* NaCl, followed by two water extractions. The four resulting supernatants were combined and dialyzed against deionized water for 48 hr at 4° C and centrifuged to separate the supernatant (albumin fraction) and the precipitate (globulin fraction) as defined by Osborne (1907). Both protein fractions were lyophilized and stored at 4° C. Alternately, ground wheat kernels were directly extracted twice with deionized water (yielding a water-extracted fraction), then extracted twice with 0.5*M* NaCl (yielding a salt-extracted fraction).

After salt and water extraction, the nonextracted material was extracted twice with 1% SDS/2% 2-mercaptoethanol. This extract we call the SDS-extracted fraction III. It contains the components of the Osborne gliadin and glutenin fractions. Alternately, the water-and-salt-nonextracted material was first extracted twice with 70% ethanol to obtain the alcohol-extracted or gliadin fraction. (By SDS-polyacrylamide gel electrophoresis of these two and subsequent ethanol extracts, we estimate that 80% of the potentially ethanol-extractable protein is obtained by two extractions. As judged by these SDS gels, the polypeptide composition of five sequential ethanol extractions is constant.) The ethanol was then removed from the gliadin fraction with N_2 gas, and the residue was lyophilized and stored at 4° C. The residual material was then extracted twice with 1% SDS/2% 2-mercaptoethanol to yield our SDS-extracted fraction IV. Because SDS/2-mercaptoethanol extracts essentially all wheat proteins (Danno et al 1974, Bietz et al 1975), our SDS-extracted fraction IV should contain the glutenin fraction.

In each of the above steps, the sample suspension was stirred gently (to prevent gluten formation) with a glass rod for 30 min, then centrifuged for 10 min at 5,000 rpm. The pellet was further extracted with another 3 ml of solvent or discarded. Each extraction step was carried out at room temperature except when otherwise indicated. Protein extracts were stored at −20° C.

Two-Dimensional Gel Electrophoresis

Samples of water- and salt-extracted fractions were concentrated for electrophoresis by precipitation with four volumes of cold acetone on a dry ice/ethanol bath for 30 min. SDS was separated

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from SDS/protein complexes in SDS-extracted fractions by diluting the sample to a final SDS concentration of 0.1% and adding four volumes of cold acetone (on dry ice/ethanol bath for 30 min) to precipitate the proteins (Hager and Burgess 1980). The precipitates were collected by centrifugation at 10,000 rpm for 20 min. The acetone-precipitated samples and the lyophilized samples (albumin, globulin, and alcohol-extracted fractions) were dissolved directly in lysis buffer (O'Farrell 1975) for the first-dimension electrophoresis. For analyzing albumin and globulin fractions, an amount of sample equivalent to the extract from one wheat kernel was used, whereas an amount of sample equivalent to the extract from one-half kernel was used for analyzing gliadin and glutenin fractions.

Either IEF or NEPHGE was used in the first dimension, with only slight modifications from the procedures given by O'Farrell (1975) and O'Farrell et al (1977). Details of the two-dimensional electrophoresis are described elsewhere (Lei et al 1983). Urea used in the first dimension was Ultra Pure (Schwartz-Mann). It was prepared fresh for each IEF or NEPHGE run. These precautions minimize the possibility of artifacts due to carbamylation of proteins by cyanate, which is generated in aqueous solutions of urea. (Harsh conditions that can lead to carbamylation are reported in Anderson and Hickman 1979). Ampholines (LKB) with pH ranges of 5–7, 7–9, and 9–11 (in 1:1:1 ratio) were used either for IEF of SDS-extracted proteins or for NEPHGE of each protein fraction in this study. In NEPHGE, total wattage of 6,000 Vhr was applied for water- and salt-extracted proteins, whereas 7,000 Vhr was applied for alcohol- and SDS-extracted proteins. Nonidet P-40 (3%) was used in the first-dimensional gels. For analysis of some acidic polypeptides in the albumin fraction, Ampholines with a pH range of 4–6 were used, and urea and Nonidet P-40 were omitted. In the second-dimensional SDS slab gels, 10% acrylamide/0.27% bis (acrylamide) was used throughout this study (unless otherwise indicated). The slab gels were stained for 4 hr in 0.2% Coomassie Blue R250/50% methanol/12% acetic acid and destained in 20% methanol/10% acetic acid.

RESULTS

Electrophoretic Analysis of Albumin, Globulin, Water-, and Salt-Extracted Fractions

Albumin and globulin fractions were prepared from the 0.5*M* NaCl and water extracts by dialysis and centrifugation. A two-dimensional analysis of the albumin fraction is shown in Figure 1, which is a composite of three electrophoregrams through which approximately 160 spots were resolved (the most abundant of which are numbered). Optimal resolution of the large majority of the spots was obtained with our standard electrophoretic system, which includes a second-dimension gel made from 10% acrylamide. Spots below numbers 12 and 13, however, were reproducibly resolved well (as in the figure) only when the second-dimension gel was made from 11% acrylamide. (In a 10% gel, these low-molecular-weight spots were frequently somewhat compressed against the electrophoretic front.) The spots in the inset in Figure 1 required use of acid ampholytes in the first dimension because they are acidic polypeptides. When the standard mixture of amypholytes was used, the spots in the inset formed a vertical banding pattern on the right side of the electrophoregram, such as that seen in Figures 3, 4, and 6 of this paper and in several of the figures in Lei and Reeck (1986). Such spots are polypeptides with a pI close to the pH at the top of the first-dimension gel and that therefore move very slowly or not at all during the first-dimension electrophoresis, or they are polypeptides that are insoluble in the first-dimension sample or lysis buffer.

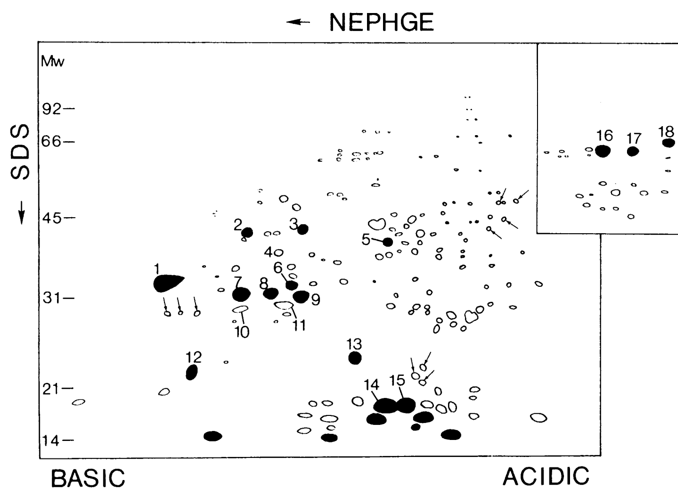

Fig. 1. Composite diagram of two-dimensional electrophoresis of wheat albumin fraction. Most of this pattern was obtained with the standard approach discussed in Materials and Methods with 6,000 Vhr in the first dimension (nonequilibrium pH gradient electrophoresis). Spots 14 and 15 and spots of similar molecular weights were resolved using a second dimension gel of 11% acrylamide. Spots 16–18 and other spots in the same area were resolved by first dimension electrophoresis (3,000 Vhr) with different Ampholines: pH range 4–6. Numbers indicate the components that are most prominent in the albumin fraction. Those polypeptides indicated with arrows are discussed in the text.

Molecular weights for the numbered spots in Figure 1 and pIs for some of those spots are given in Table I. The pIs were not determined from the electrophoregram in Figure 1, but from gels in which the first dimension was carried to equilibrium (i.e., performed as IEF). Only in such gels is a polypeptide located at a pH equal to its pI, and only for such gels is it meaningful to show a pI scale. Thus such a scale is given only for Figure 4. Several of the polypeptides that are numbered in Figure 1 are sufficiently basic so as to make estimation of their pIs difficult. (O'Farrell et al [1977] contains a discussion of this point.)

The globulin fraction (Fig. 2) is very distinct from the albumin fraction. For convenience we rather arbitrarily divided many of the globulin polypeptides into four groups: globulin group I (GgI), globulin group II (GgII), globulin group III (GgIII), and globulin group IV (GgIV). GgI and GgII both contain several subgroups of polypeptides that have the same molecular weights but small (apparently single) charge differences. The relative molecular weights of some globulin polypeptides are shown in Table II. In addition to the four globulin groups, other polypeptide spots, including some of low molecular weight (around 20,000), are also seen in the electrophoregrams but are less intense. Electrophoretic analyses of fractions from manually dissected kernels indicate that GgI polypeptides are nearly or totally absent from embryo, but present in bran and endosperm. GgII polypeptides are present in bran, endosperm, and embryo. GgIII polypeptides are nearly absent in bran and endosperm, but prominent in the embryo. GgIV polypeptides are apparently restricted to embryo (results not shown).

Some spots are found in more than one solubility fraction. Three components near polypeptide 15 (indicated by 3 arrows) were

TABLE I
The Relative Molecular Weights of Selected Albumin Polypeptides

Polypeptide	Molecular Weight	pI
1	33,000	ND[a]
2, 3	42,000	ND
4	39,000	ND
5	40,000	6.7
6	33,000	ND
7, 8	32,000	ND
9	31,000	ND
10, 11	30,000	ND
12	22,000	ND
13	23,000	6.9
14	18,000	6.8
15	18,000	6.6
16	64,000	5.5
17	64,000	5.2
18	66,000	4.8

[a]ND = not determined.

present in both the albumin and globulin fractions (Figs. 1 and 2). Also four polypeptides indicated by arrows on the right side of Figure 1 are present in the gliadin and glutenin fractions to be discussed.

Samples of water- and salt-extracted proteins obtained following water extraction were also analyzed (results not shown). In general, the two-dimensional electrophoregrams of the water-extracted fraction and salt-extracted fraction were similar but not identical to those of albumin fraction (Fig. 1) and globulin fraction (Fig. 2), respectively. Some polypeptides of the albumin fraction were present in the salt-extracted fraction, apparently having been incompletely extracted by water even though they are water-soluble. Included among these were polypeptides 1, 6, 7, and 8.

Electrophoretic Analysis of Water-and-Salt-Nonextracted Proteins

The water-and-salt-nonextracted material of wheat kernels was extracted with 1% SDS in the presence of 2% 2-mercaptoethanol (SDS-extracted fraction III) and resolved on two-dimensional gels (Figs. 3 and 4). Both NEPHGE (Fig. 3) and IEF (Fig. 4) were used in the first dimension of the electrophoretic analyses. The sample preparation was intended to produce the combined gliadin and glutenin fractions, but several components of the albumin and globulin fractions were faintly visible on the electrophoregrams: The minor polypeptide spots right below the dotted lines (Figs. 3 and 4) are residual GgI. The four spots indicated by arrows on the right-hand side of Figure 3 are residual albumin components (as in Fig. 1).

From their molecular weights and relative abundance, we judge that the spots above the horizontal dotted lines on both Figures 3 and 4 correspond to the "high-molecular-weight glutenins" of Payne et al (1982). The molecular weights of these polypeptides are above 85,000, and most are neutral or basic polypeptides. The molecular weights of the majority of the polypeptides found in SDS-extracted fraction III are in the range of 30,000–55,000. There are also three polypeptides of somewhat lower molecular weight, indicated by arrowheads in Figure 3. It is interesting to note that one spot, indicated by an arrowhead in Figure 4, is purple-pink or sometimes very faint (possibly due to the fading after destaining). It can be a useful landmark in comparing gel patterns.

TABLE II
The Relative Molecular Weights of Globulin Polypeptides

Polypeptide	Average Molecular Weight
GgI	70,000
GgII	60,000
GgIII	42,000
GgIV	42,000

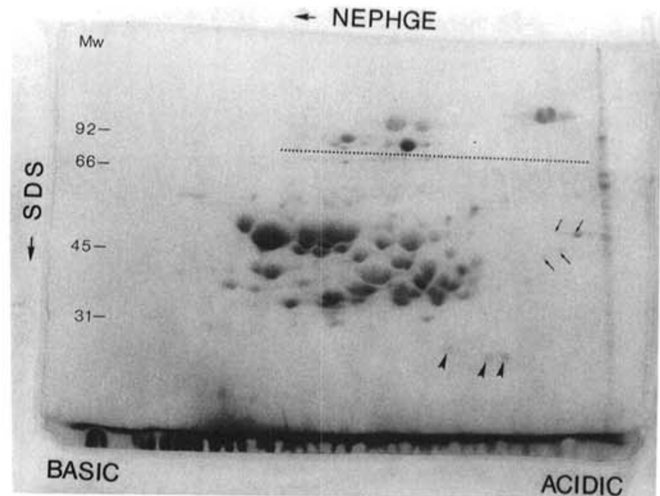

Fig. 3. Two-dimensional electrophoresis of water-and-salt-nonextracted proteins: extracted with 1% SDS/2% 2-mercaptoethanol (sodium dodecyl sulfate-extracted fraction III). A total of 7,000 Vhr was applied in the nonequilibrium pH gradient electrophoresis. The majority of the polypeptides in this fraction have molecular weights of about 30,000–55,000. The spots above the horizontal dotted line having molecular weights above 85,000 apparently correspond to the "high-molecular-weight glutenins" of Payne et al (1982). Arrowheads point to three faint polypeptides that occur in both the gliadin and glutenin fractions and that we have not included in group A, B or C. Arrows indicate the residual components of albumin fraction. The components just below the horizontal dotted line are residual GgI.

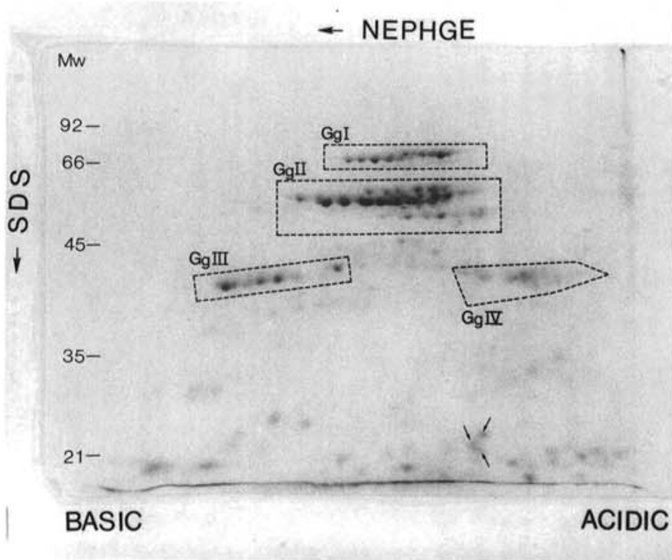

Fig. 2. Two-dimensional electrophoresis of wheat globulin fraction. A total of 6,000 Vhr was applied in the nonequilibrium pH gradient electrophoresis. The globulin polypeptides are designated into four groups indicated as GgI, GgII, GgIII, and GgIV. Polypeptides indicated with arrows are discussed in the text.

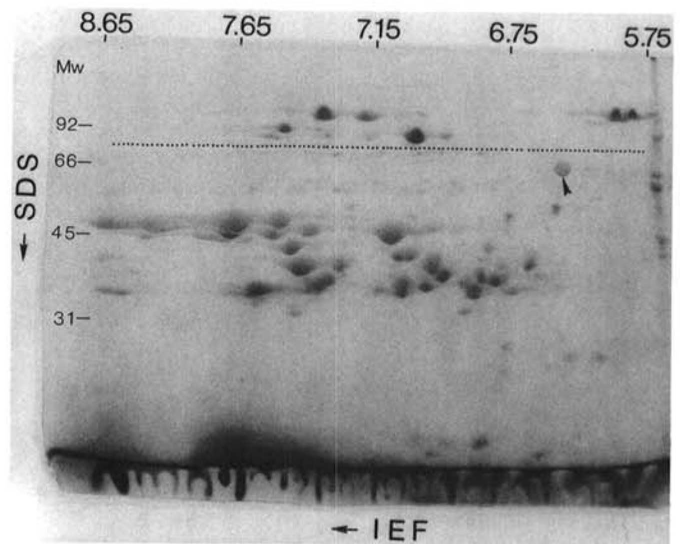

Fig. 4. Two-dimensional electrophoresis of water-and-salt-nonextracted proteins (sodium dodecyl sulfate-extracted fraction III), with isoelectric focusing in the first dimension. Polypeptides above the horizontal dotted line having molecular weights above 85,000 apparently correspond to the "high-molecular-weight glutenins" of Payne et al (1982). Arrowhead points to a polypeptide that is purple-pink.

The water-and-salt-nonextracted material was also fractionated differentially with 70% ethanol and 1% SDS/2% 2-mercaptoethanol to obtain the gliadin fraction (alcohol-extracted proteins) and a final fraction (SDS-extracted fraction IV) that should contain all residual proteins and include the glutenin fraction (which would classically be obtained by alkali or acid extraction after alcohol extraction). The gliadin fraction and SDS-extracted fraction IV were analyzed by two-dimensional gels (Figs. 5 and 6), with NEPHGE in the first dimension. Almost every spot in Figure 5 (gliadin fraction) can be found in Figure 6 (SDS-extracted fraction IV), and most of the spots in Figure 6 are present in Figure 5. Among polypeptides common to the two fractions, those that are relatively intense in Figure 5 are relatively faint in Figure 6 (and vice versa). We divide these major storage proteins of wheat kernels into three groups (A, B, and C) on the basis of electrophoretic mobility on SDS gels and extractability in 70% ethanol. The polypeptides of group A are of relatively high molecular weight (above 85,000). They are above the horizontal dotted line in Figure 6 and are indicated by arrowheads in the upper portion of Figure 5. Group B polypeptides (with molecular weights ranging from 33,000 to 52,000) are shown inside the dotted line of Figure 6 and inside the left dotted line of Figure 5, indicated by "B." The polypeptides of group C (with molecular weights ranging from 30,000 to 55,000) are shown inside the right dotted line of Figure 5, indicated by "C." Group C polypeptides are more readily extracted with 70% ethanol than those of group B. Group B polypeptides are more basic than those of groups A and C, and many group B polypeptides are not recovered on the electrophoregrams where IEF is used as the first dimension (Lei 1984).

The results shown in Figures 5 and 6 indicate that the gliadin and glutenin fractions share many individual polypeptides. This is evidently an inherent property of the system that cannot be overcome by performing the "right" number of ethanol extractions. This can be seen as follows. When only two ethanol extractions are performed (as in preparing the sample for Fig. 5), the group B polypeptides, which are the predominant components in the glutenin fraction, also occur as prominent components in the gliadin fraction. Thus, no minimum amount of extraction with ethanol will prevent the group B polypeptides from occurring in both the gliadin and glutenin fractions. On the other hand, exhaustive ethanol extraction (which could be used to minimize the occurrence of the group C polypeptides in the glutenin fraction) simply increases marginally the occurrence of the group B polypeptides in the gliadin fraction (while still leaving those polypeptides as the predominant components of the glutenin fraction).

DISCUSSION

The two-dimensional gel electrophoresis described by O'Farrell (IEF/SDS-PAGE or NEPHGE/SDS-PAGE) has been used before to analyze wheat kernel proteins (Brown et al 1979, 1981; Brown and Flavell 1981; Holt et al 1981; Jackson et al 1983). Our studies differ from earlier analyses in characterizing and comparing all four Osborne solubility fractions. For such comparisons the O'Farrell approach to two-dimensional electrophoresis is particularly appropriate, because the solvents in both dimensions of the scheme provide excellent conditions for generalized protein solubilization. For less general purposes, it is entirely possible that other electrophoretic approaches would be as good or even superior to O'Farrell's two-dimensional gels. For instance, a powerful low pH (aluminum lactate) one-dimensional system is an excellent method for analyzing the gliadin fraction of wheat (Lookhart et al 1982, Khan et al 1983). (The one-dimensional lactate system seems to resolve fewer components than does the O'Farrell approach in our hands. The lactate electrophoresis system, when combined with SDS-PAGE in a second dimension [Payne et al 1982], is possibly more powerful for the gliadin fraction, however, than is the O'Farrell approach.) To reiterate, the O'Farrell two-dimensional electrophoresis is the approach most appropriate for the comparison of the four Osborne solubility fractions, because their components span a wide range of solubilities and other physical properties (isoelectric points and molecular weights).

Each Osborne solubility fraction has been known for some time to be quite heterogeneous. As increasingly powerful analyses are performed on heterogeneous protein fractions (from whatever source), it is typical to resolve more and more components. Our work is an example of that trend, particularly as regards the complexities of the albumin and globulin fractions. For the gliadin fraction and our glutenin-containing SDS fraction IV, our analyses revealed only modestly greater complexity than had been previously recognized. (The improvement in analysis is more apparent for the glutenin fraction than the more intensively studied gliadin fraction.) It is interesting to note that our two-dimensional analyses of the gliadin fraction reveals more spots than there are peaks in the chromatograms obtained by high-performance liquid chromatography (HPLC) of Bietz (1983). Hence, as impressive as that HPLC approach is, it apparently falls short of resolution of all gliadin components.

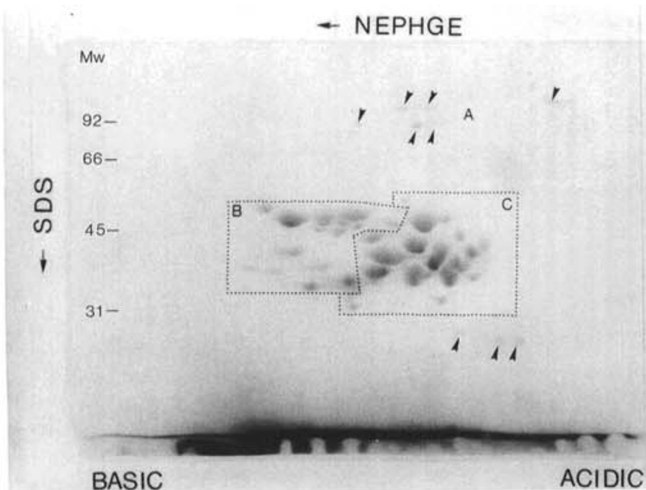

Fig. 5. Two-dimensional electrophoresis of alcohol-extracted protein fraction (after water and salt extractions). A total of 7,000 Vhr was applied in the nonequilibrium pH gradient electrophoresis. Arrowheads near A indicate faint group A polypeptides. Arrowheads below the group C polypeptides indicate three faint polypeptides that occur in both the gliadin and glutenin fractions.

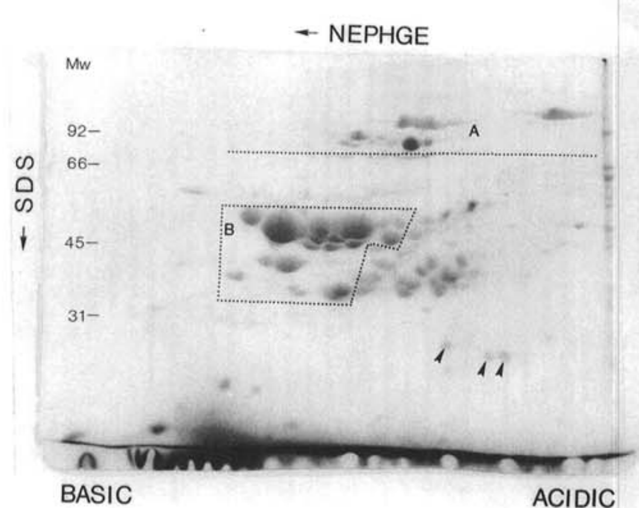

Fig. 6. Two-dimensional electrophoresis of sodium dodecyl sulfate-extracted fraction IV (after water, salt, and alcohol extractions). A total of 7,000 Vhr was applied in the nonequilibrium pH gradient electrophoresis. Arrowheads below the group C polypeptides indicate three faint polypeptides that occur in both the gliadin and glutenin fractions.

A generalization with apparently wide validity is developing with regard to storage proteins in cereals and legumes. Most often, a storage protein is not a single chemical species but instead a family of related polypeptides. (By related, we mean having evolved from a common ancestral protein and therefore exhibiting sequence similarity and sharing three-dimensional folding patterns.) It is clear, for instance, that polypeptides of the wheat gliadin fraction are related to each other (e.g., γ_1-, β_5-, and α_{8-12}-gliadin components have the same N-terminal amino acid sequence, and γ_2- and γ_3-gliadin components are similar to γ_1-, β_5-, and α_{8-12}-gliadin components in N-terminal amino acid sequence [Bietz et al 1977]). Our electrophoretic analysis of the globulin fraction of wheat suggests that the occurrence of protein families is not limited to the most abundant components of kernels. In at least two groups of the globulin fraction (GgI and GgII in Fig. 2) there are sets of 6–10 spots all having the same SDS-PAGE mobility but differing slightly in mobility in the first dimension. The differences between adjacent spots seem consistent with single charge differences. It is likely that such a set of spots (of essentially identical molecular weights but slightly different pIs) constitutes a family of related proteins.

Our analyses of the wheat gliadin and glutenin fractions leads us to believe, as others have previously stated (Bietz and Wall 1975, Miflin and Shewry 1979, Payne and Corfield 1979, Field et al 1982), that the terms gliadin and glutenin, as commonly used, are seriously flawed. In the context of wheat kernel proteins, the major shortcoming of the Osborne solubility terms is that, to a first approximation, all components of the wheat gliadin fraction occur in the glutenin fraction and vice versa. Any given component is simply more abundant in one fraction than in the other. This is an inherent feature of the wheat kernel proteins and cannot be eliminated by choosing a correct number of ethanol extractions. In common usage, a component of the gliadin fraction is called a gliadin. Such a protein, however, could also be called a glutenin by virtue of its occurrence in the glutenin fraction. Many individual proteins could thus be given two different names.

The gliadin fraction can be a distinct operational entity, as can the glutenin fraction. The individual components that constitute these fractions are not distinct, however, and therein lies the problem. Other separation methods, such as gel permeation chromatography, may give more distinct subpopulations of wheat kernel proteins than those produced by the Osborne fractionation scheme. To use the terms gliadin and glutenin to refer to chromatographic fractions (without reference to the Osborne scheme) simply introduces more ambiguity.

We therefore believe that gliadin and glutenin should be used in an operational sense to refer to fractions—the gliadin fraction and the glutenin fraction—obtained essentially as described by Osborne (1907). Individual proteins not extracted by water or salt (proteins commonly called gliadins or glutenins) should instead be called water-and-salt-nonextracted proteins (for accuracy) or gluten proteins (for simplicity). An individual gluten protein could be assigned a name that includes its pI and molecular weight. Thus GP(50, 7.3) could designate a gluten protein of 50,000 molecular weight and a pI of 7.3. Such an operationally based scheme would provide a systematic means of naming and cataloging this large group of interrelated wheat proteins, members of which will increasingly be studied by protein chemists and molecular biologists as single chemical species, and which therefore is in urgent need of a nomenclature system that is operational and unambiguous.

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