WHEAT AND WHEAT IMPROVEMENT

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Origins and Analyses of Genes and Genomes in Wheat and Its Relatives

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The endosperm of common wheat (*Triticum aestivum* L.) contains a great number of nonenzymatic storage proteins that are the components of gluten, one of the most intricate naturally occurring protein complexes. Gluten may be subdivided, on the basis of differential solubility, into gliadin and glutenin, as described by Osborne (1907). Gliadin is a fraction, largely monomeric, that is soluble in alcohol-containing solutions. Glutenin consists of numerous polypeptides that are further associated covalently and noncovalently into high-molecular-weight (MW) complexes, which are insoluble in alcohol but may be soluble in solutions containing dilute acids or alkali, denaturants, detergents, or disulfide-reducing agents. In addition, some albumin and globulin proteins normally soluble in water or dilute salt solutions may also be incorporated into gluten during extraction or mixing. Several excellent reviews consider gluten’s composition, properties, interrelationships of its constituent proteins, and use in genetic analysis (Kasarda et al., 1976; Miflin and Shewry, 1979; Wall, 1979; Wrigley, 1982; Kasarda et al., 1984a; Konarev et al., 1979; Stegemann and Pietsch, 1983).

Gluten is likely to contain several hundred different polypeptides, many being homologous. This heterogeneity is in part due to gene duplication followed by mutation; it is also due to the hexaploid nature of wheat, which evolved from three separate but related diploid species. Thus, each homoeologous chromosome group contains structural genes having similar positions within each genome. Since few constraints regulate gene expressions, their translated proteins serve as accurate “fingerprints” and can be used to identify genotypes, to provide markers of genes, and to determine genetic and evolutionary relationships.
Several methods are now extensively used to determine relationships among proteins within a genotype and to differentiate cultivars within a species; these methods may also reveal relationships among species and genera, since mutational rates can be used to predict evolutionary divergence. In wheat breeding, these proteins may provide markers for specific quality characteristics. This review will consider the application of electrophoresis, high-performance liquid chromatography, immunochemical procedures, and protein sequence analysis to wheat genetic studies and improvement through breeding.

5D-1 GEL ELECTROPHORESIS

In gel electrophoresis, proteins migrate in an electric field through a porous matrix, typically composed of polyacrylamide or starch. Mobilities are directly related to charge and inversely related to size; however either size or charge can be suppressed. Thus, in isoelectric focusing, proteins migrate in a pH gradient to positions of electric neutrality that are independent of MW. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), SDS masks protein charge, and thus separations depend only on size. The following sections review the value of gel electrophoresis for wheat cultivar identification and aneuploid analysis, and for determining evolutionary relationships and predicting quality.

5D-1.1 Cultivar Identification

Where wheat is marketed as pure cultivars, cultivar identification may be necessary to determine or assure quality. It also may be desirable to identify genotypes used in breeding programs. Cultivar identification is now possible through electrophoresis of gliadin, glutenin, albumin plus globulin, and total protein extracts.

5D-1.1.1 Gliadins

In 1959, Jones et al. demonstrated that moving-boundary electrophoresis in aluminum lactate-lactic acid buffer, pH 3.2, could resolve gluten into several fractions. Combining this buffer with zone electrophoresis in starch gels (Smithies, 1955) significantly improved gliadin resolution (Elton and Ewart, 1960; Woychik et al., 1961; Elton and Ewart, 1962), permitting cultivar differentiation. Wheat cultivar identification by starch gel electrophoresis (SGE) of gliadins has become a widely used method (Autran and Bourdet, 1975; Wrigley et al., 1982a). (Typical results are shown in Fig. 5D-1.) Twenty to 30 bands are normally revealed by this method; on the basis of their presence, absence, and intensity, keys have been developed for identifying cultivars. Computer-assisted identification also is possible. Gliadins are well suited for cultivar identifi-
cation because of their heterogeneity, monomeric nature, ease of extraction, stability, and near-constant expression under various environmental conditions. Starch gel electrophoresis of gliadins can be performed on flour samples or on single kernels. Consequently, SGE of gliadins is widely used for cultivar identification.

Starch gel electrophoresis uses simple equipment and a nontoxic support, but resolution may vary and gels are difficult to prepare reproducibly. It is easier to prepare reproducible gels from acrylamide, and PAGE of gliadin is now widely used for wheat cultivar identification (Bushuk and Zillman, 1978; Wrigley et al., 1982a). (An example is shown in Fig. 5D-2.) Separations are analogous to those obtained by SGE, but resolution is generally better. Several excellent alternative PAGE procedures have been proposed (Tkachuk and Mellish, 1980; Lookhart et al., 1982; Khan 1982; Khan et al., 1983). Most separations are performed in aluminum lactate buffer at acidic pH, but good resolution can also be obtained using acetic acid/glycine buffer (Maier and Wagner, 1980). Alkaline
Fig. 5D–2. Polyacrylamide gel electrophoresis (PAGE) patterns of gliadins from nine wheat cultivars. From Bushuk and Zillman (1978).

buffers (Hussein et al., 1977; Hussein and Stegemann, 1978; Ohms, 1980b), though used less frequently, also may give good separations: in particular, acidic and alkaline buffers may be effectively combined in a two-dimen­sional horizontal PAGE procedure (Mecham et al., 1978), in which sam­ples for alkaline PAGE are loaded in the middle of the gel to prevent cathodic components from being lost.

Gradient gel electrophoresis of gliadins also permits cultivar identification. In this procedure, gel concentration increases with migration distance, mobilities decrease, and zones are sharpened (du Cros and Wrig­ley, 1979; Wrigley et al., 1982a) (Fig. 4D–3). Advantages of the method include speed, convenience, and commercial availability of gels; repro­ducible homemade gels may be somewhat difficult to prepare.

Isoelectric focusing (IEF), first applied to gliadin by Wrigley (1968), also differentiates wheat cultivars (du Cros and Wrigley, 1979; Wrigley et al., 1982a). Isoelectric focusing separations, which depend only on charge, thus complement other electrophoresis procedures; however, the cost of carrier ampholytes may restrict routine use of IEF.

SDS-PAGE of gliadin may also be useful for cultivar identification (Shewry et al. 1978). SDS-PAGE complements SGE, and may differen­tiate some cultivars that appear identical by SGE.

Two-dimensional procedures provide the best available electrophore­tic resolution of gliadin. Nearly any two procedures may be combined, but most separations use either IEF followed by SGE (Wrigley, 1970; Wrigley and Shepherd, 1973; du Cros and Wrigley, 1979) or PAGE at two pHs (Mecham et al., 1978). Usually 40 to 50 gliadins resolve, far more than in any one-dimensional method, and thus two-dimensional methods may be able to identify cultivars that appear identical in one-dimensional procedures. Two-dimensional electrophoresis is perhaps too complex for routine use, but is invaluable for special applications.
Wheat cultivar identification by electrophoresis of glutenin is less common than by electrophoresis of gliadin. In part, this is because glutenin must be converted to its subunits before electrophoresis, through reduction of disulfide bonds in the presence of either strong denaturants (e.g., 8 M urea) or detergents (e.g., SDS). Resulting subunits can be classified as being high-MW (ethanol-insoluble) or low-MW (ethanol-soluble). Also, glutenin's subunits may be difficult to separate because of their high MWs, solubilities, and associative tendencies. Nevertheless, coding of many high-MW glutenin polypeptides is different from that of most gliadins, and glutenin subunit compositions are nearly constant for any cultivar. Thus, if gliadin electrophoresis does not differentiate genotypes, analysis of glutenin subunits may succeed.

Differences in glutenin subunit composition clearly exist among cultivars (Elton and Ewart, 1966; Huebner, 1970). Resolution of glutenin subunits by SGE is relatively poor, however, and their isolation is difficult. The application of SDS-PAGE (Bietz and Wall, 1972) permitted glutenin subunits to be more easily characterized. Nevertheless, resolution originally was not sufficient to differentiate most cultivars: subunit compositions of glutenins isolated from single hexaploid wheat kernels for 75 out of 80 cultivars were similar (Bietz et al., 1975). Five cultivars, however, including 'Nap Hal', differed markedly in high-MW (100 000–130 000 daltons) subunit composition. Because they have a much higher
MW than all other wheat polypeptides, high-MW glutenin subunits in easily obtained total protein extracts also can be analyzed by SDS-PAGE.

By application of higher resolution SDS-PAGE, considerably more variability has been observed among high-MW glutenin subunits (Payne et al., 1981b) (Fig. 5D–4). Similar studies have been performed by Lawrence and Shepherd (1980) and by Galili and Feldman (1983b). At least 20 distinct high-MW subunits have been identified in wheat. Generally, four to five high-MW subunits are present in any cultivar; polymorphism among these subunits, arising from expression of different alleles, permits cultivar identification (du Cros et al., 1980; Wrigley et al., 1982a). These differences are also apparent in total protein extracts, simplifying sample preparation. Thus, SDS-PAGE of high-MW glutenin subunits may be useful for cultivar identification, particularly when cultivars have similar gliadin compositions.

5D-1.1.3 Albumins and Globulins

Electrophoresis of albumins and globulins may also be useful in wheat cultivar differentiation, particularly if gliadin and glutenin subunits are
similar, because albumins and globulins are coded by different chromosomal loci. Elton and Ewart (1964) showed significant qualitative and quantitative differences among albumins of several wheat cultivars by SGE. Doekes (1968) examined 80 wheat cultivars by SGE and densitometry, dividing them into five subgroups reflecting genetic relationships. Ohms (1980b) demonstrated that PAGE of albumins and globulins extracted in the presence of 2-mercaptoethanol could also differentiate some genotypes. A similar procedure was reviewed by Wrigley et al. (1981), who noted, as did Johnson (1972), that electrophoresis of soluble proteins may be more useful for comparisons of species than of cultivars.

5D-1.1.4 Total Proteins

SDS-PAGE patterns of total protein extracts can also differentiate wheat cultivars (Fullington et al., 1980); both qualitative and quantitative differences exist between genotypes. Galili and Feldman (1983b) also used high-resolution SDS-PAGE to compare wheats (Fig. 5D-5). Much quali-

![MW markers](image)

**Fig. 5D-5.** High-resolution sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total protein extracts from the hexaploid wheat cultivars (a) Chinese Spring, (b) Merav, (c) Florence Aurore, (d) Ribeiro, (e) Lackish, (f) Minam, (g) Jaral, (h) Tanon, (i) Inia 66, (j) Sonora 66, (k) TAA19, (l) Hazera 112, (m) Hazera 776, (n) Mabrouk, (o) Selkirk, and (p) WA 6389. Known molecular weight (MW) markers were fractionated in a parallel lane, with apparent MW indicated on the left. Specific polypeptides are identified within the gel. From Galili and Feldman (1983b).
itative and quantitative variation was observed both in high-MW subunits (glutenin) and in lower-MW subunits. Clearly, this may be a valid alternative to other electrophoretic procedures for cultivar identification.

5D-1.2 Locations of Genes Coding Gluten Proteins

The heterogeneity of wheat protein complicates many studies, but it may also be used advantageously in genetic studies. Determination of chromosomal control may clarify synthesis, interrelationships, and expression of proteins; such proteins may then be used as markers (either causative or closely linked) of desirable or undesirable characteristics in breeding. Such an application is possible largely because of the availability of wheat aneuploids pioneered in 'Chinese Spring' by E.R. Sears (1954), and because of high-resolution electrophoretic methods. Others have recently reviewed genetic analyses of wheat proteins by electrophoresis (Wrigley, 1982; Garcia-Olmedo et al., 1982; Porceddu et al., 1983; Payne et al., 1984b); this section will focus on some of the landmark studies in this area.

5D-1.2.1 Gliadins

In one of the first uses of electrophoresis to characterize wheat aneuploids, Boyd and Lee (1967) noted changes in gliadin SGE patterns of ditelocentric vs. euploid lines of Chinese Spring; when one and the same arm of each of the 1D chromosome pair was removed, two slow-moving ω-gliadins disappeared, showing that they represent heritable characteristics. Shepherd (1968) subsequently examined all nullisomic-tetrasomic and ditelocentric Chinese Spring lines: 9 of 17 major bands were accounted for by the absence of genes on the short arms of chromosomes 1A, 1B, 1D, 6A, and 6D; chromosome dosage effects observed for other bands suggested that one-dimensional SGE does not resolve all gliadins, since some bands were coded for by multiple genes on different chromosome arms. Wrigley and Shepherd (1973) combined IEF with SGE in a two-dimensional procedure, and resolved Chinese Spring gliadins into 46 components, most coded by genes on chromosome groups 1 and 6 (Fig. 5D-6). Other high-resolution, two-dimensional electrophoretic methods may also demonstrate chromosomal control: Mecham et al. (1978) used PAGE first at pH 3.2 and then at pH 9.2 to separate gliadins. Brown et al. (1979) combined IEF with SDS-PAGE to obtain high-resolution separations. In a variation of this method, Jackson et al. (1983) and Payne et al. (1984b) combined separations by both IEF and non-equilibrium pH-gradient electrophoresis (NEPHGE) in the first dimension with SDS-PAGE in the second (Fig. 5D-7), giving the highest-resolution electrophoretic separations of wheat endosperm proteins obtained to date. These studies conclusively demonstrate that genes coding most α and β gliadins are on the short arms of homoeologous group 6 chromosomes, whereas most ω- and γ-gliadin genes are on the short arms of chromosomes 1A, 1B, and 1D.
Electrophoretic analysis of gliadins from aneuploids has revealed considerable additional genetic information. One of the most significant concepts is that gliadins are inherited as blocks of components, suggesting that gliadin genes are clustered in discrete loci, each of which may vary in composition (Sozinov and Poperelya, 1980). Each group 1 and 6 chromosome has only one complex locus, so that loci are inherited independently, causing variation that permits cultivar differentiation: this independent inheritance has led to a proposed nomenclature based on gliadin blocks. Mecham et al. (1978) and Branlard (1983) have also observed that blocks of closely linked genes code for gliadin proteins.

Although most original genetic studies of gliadins were done in Chinese Spring, various substitution lines also permit analysis of other hexaploid cultivars (Mecham et al., 1978; Brown et al., 1981; Kasarda et al., 1984a). These studies confirm the groups 1 and 6 coding observed in Chinese Spring. Similarly, durum (Triticum turgidum L.) D-genome disomic-substitution lines, each having a different D-genome chromosome pair substituted for homoeologous A- or B-genome chromosomes, have made possible chromosomal location of genes for most durum gliadins resolved by one- or two-dimensional electrophoresis (Joppa et al., 1983b; du Cros et al., 1983).

5D-1.2.2 Glutenins

Because of their higher MWs, associative tendencies, and poor solubilities, glutenin subunits may separate poorly upon SGE or PAGE. It
was only through SDS-PAGE (Bietz and Wall, 1972) that high-MW glutenin subunits were found to be coded by genes on the long arms of chromosomes 1D and 1B (Orth and Bushuk, 1974; Bietz et al., 1975). Because these subunits are unique to glutenin and because chromosomes 1B and 1D have been associated with bread wheat quality (Welsh and Hehn, 1964; Morris et al., 1966), Bietz et al. (1975) examined numerous hexaploid cultivars: some variability among high-MW subunits was noted, but most cultivars appeared similar. These results were subsequently extended (Payne et al., 1980; Lawrence and Shepherd, 1981b; Galili and Feldman, 1983a), using higher resolution SDS-PAGE. Additional heterogeneity became apparent among cultivars, and high-MW glutenin subunits were found to be inherited in blocks, as are gliadins, and to be controlled by presumably homologous gene loci on the long arms of chromosomes 1A, 1B, and 1D (Lawrence and Shepherd, 1981a; Payne et al., 1982; Galili and Feldman, 1983b). Crosses between wheats having different high-MW glutenin subunit compositions have revealed at least 21 allelic variants at glutenin subunit loci Glu-A1, Glu-B1, and Glu-D1 (Payne et al. 1984b). Glutenin subunit inheritance in F1 and F2 generations is co-dominant (Burnouf et al., 1981; Burnouf et al., 1983b), so subunits are expressed whatever their gene dosage in the endosperm. As discussed below, specific alleles are associated with bread-making quality (Burnouf and Bouriquet, 1980; Payne et al., 1980), permitting screening of early generation progeny for desirable characteristics.

Glutenin's ethanol-soluble low-MW subunits (Bietz and Wall, 1980) have recently been shown to be coded by genes on the short arms of chromosomes 1A, 1B, and 1D (Jackson et al., 1983). These polypeptides may be of considerable importance, since in durum wheat the presence or absence of a low-MW glutenin subunit is as closely related to pasta quality as are the more commonly used gliadin marker protein bands 42 and 45 (Payne et al., 1984c).

Although allelic variation among glutenin subunits is frequently apparent by one-dimensional SDS-PAGE, two-dimensional separations provide additional information. In characterizing glutenin, Brown et al. (1979, 1981) and Brown and Flavell (1981) used combined IEF and SDS-PAGE, and Jackson et al. (1983) and Payne et al. (1984b) also used nonequilibrium pH-gradient electrophoresis (see Fig. 5D-7). These techniques are powerful tools for selection and improvement of wheat lines.

5D-1.3 Use of Electrophoresis to Study Genetic Relationships Among Genera and Species

Early studies using protein electrophoresis to assess phylogenetic affinities among wheat genera and species were performed by B.L. Johnson and co-workers. Proteins may provide a more direct measure of gene homology than morphological relationships (Johnson and Hall, 1965).
As gene mutations accumulate within a species, charges of proteins also may change, affecting their mobilities. Since polyploid genomes represent gene systems transmitted relatively intact from donor species, protein spectra should indicate genome donors, as well as homologies. Johnson (1972) thus analyzed gliadin or albumin proteins by PAGE combined with densitometry, and determined correlation coefficients between corresponding spectral points to estimate genetic relationships. His conclusions generally agreed with other data, while suggesting novel relationships. In addition, comparison of triticale (X Triticosecale Wittmack) with wheat and rye (Secale spp.) confirmed that electrophoretic spectra of amphiploids are the sum of spectra of parental proteins.

Considerable care, however, must be used in such comparisons. For example, intraspecific variability in gliadin composition may be as great as interspecific variation (Boyd et al., 1969); an obvious example is variability among T. aestivum gliadins, which permits cultivar identification. However, generally there is little intraspecific variation among albumins, making them more useful than gliadins for comparing species (Wrigley...
et al., 1982a). In crosses between known parents, however, gliadins also provide suitable markers. A few examples of such methods will be noted.

Studies of wheat and rye have shown that characteristic markers exist for specific *Triticum* and *Secale* chromosomes (Shepherd and Jennings, 1971), and that triticale's proteins are simply inherited from its parents (Chen and Bushuk, 1970). This permits, for example, electrophoretic determination of amounts of wheat and rye in a mixture (McCausland and Wrigley, 1976). Similarly, electrophoretic analysis of proteins characteristic of the D genome of *T. aestivum* permits determination of amounts of common wheat in pasta products (Feillet and Kobrele, 1972). Differential staining of albumins and globulins may also help to reveal evolutionary relationships and pedigrees of species and genera (Caldwell and Kasarda, 1978). SDS-PAGE is also useful to demonstrate and clarify evolutionary relationships in the Triticinae (Preston et al., 1975), and to reveal marker proteins characteristic of specific chromosomes in barley (*Hordeum* spp.), rye, *Triticum umellulatum*, and *Agropyron elongatum* (Lawrence and Shepherd, 1981a). SDS-PAGE of total protein extracts, in combination with densitometry, may also reveal relationships among diploid and polyploid *Triticum* (including *Aegilops*) spp. (Cole et al., 1981). Thus, there are numerous ways to use electrophoresis of seed proteins to mark genetic systems and to define genotypes (Konarev et al., 1979).

### 5D-1.4 Predication of Wheat Quality by Electrophoresis

To a large degree, the functional and nutritional qualities of wheat are related to its proteins. Since all protein fractions are extremely heterogeneous, high-resolution techniques such as electrophoresis may yield much qualitative and quantitative information, and it is likely that such data may be related to quality. Such relationships already have been found and are likely to increase (see Wrigley, 1982, and Khan, 1982, for reviews). This section will review recent results of relating electrophoresis to wheat quality.

Damidaux et al. (1978) first noted a consistent relationship between two durum gliadins, bands 45 and 42, and gluten viscoelasticity (Fig. 5D–8). Cultivars having gliadin 45 typically have cooking quality superior to cultivars with gliadin 42. These results were confirmed by Kosmolak et al. (1980). Gliadins 42 and 45 were found to be coded by two codominant alleles of a single gene on chromosome 1B (Damidaux et al. 1980). Computer-based pattern analysis could also demonstrate relationships of gliadin electrophoregrams to dough strength and weakness (du Cros et al., 1982). In addition, improved resolution of two-dimensional electrophoresis accentuated the heterogeneity of these proteins (du Cros et al., 1983). Thus, electrophoresis can be valuable in screening for grain quality. It is important to realize, however, that observed associations may be due to close proximity of genes coding gluten-protein markers to other genes that confer quality, and not to any direct contribution of specific poly-
peptides to quality (Wrigley, 1982). For example, an intermediate-MW durum-glutenin subunit (Autran, 1981) is more closely associated with cooking quality than are the gliadin marker proteins 45 and 42 (Payne et al., 1984c).
Much evidence now suggests that hexaploid-wheat protein fractions may also be related to bread-making quality. For example, blocks of genes that code gliadin proteins may be related to characteristics such as sedimentation volume (Sozinov and Poperelya 1980). Furthermore, there are significant correlations between specific gliadin electrophoretic bands and characteristics such as hardness and dough strength (Branlard and Rousset, 1980; Wrigley et al., 1981, 1982b, 1982c; Pogna et al., 1982). Detection of such relationships is greatly facilitated through the use of computers. As noted above, however, such correlations do not necessarily indicate cause and effect, but rather may be due to close linkage of gliadin genes to other genes actually associated with quality, or to a relationship of electrophoretic pattern to pedigree (which in turn may relate to quality). Other proteins also may be associated with good baking quality: for example, two proteins extracted by Tris-glycine buffer, pH 8.9, in the presence of 2-mercaptoethanol occur preferentially in cultivars with good baking quality (Ohms, 1980a).

One of the most intriguing correlations between protein composition (as revealed by electrophoresis) and bread-making quality concerns glutenin, the strength protein of wheat. Payne et al. (1979, 1981a) identified specific high-MW glutenin subunits that correlated with quality; similar results were obtained by Burnouf and Bouriquet (1980) and by Moonen et al. (1982). Much allelic variation exists at the Glu-A1, Glu-B1, and Glu-D1 loci (Fig. 5D-9), and variants differ in their relationship to quality. These studies are currently useful in directing breeding strategies, and increased knowledge of sequences of these subunits may soon reveal the true relationship between glutenin structure and functionality (Shewry et al., 1984).

5D-2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

In liquid chromatography, solutes dissolved in a flowing-liquid mobile phase pass through columns packed with solid supports: separations are based on relative interactions of solutes with column packings. Column techniques have provided good separations of wheat proteins based upon ionic interactions between solutes and support (ion-exchange chromatography) or upon solute penetration into or exclusion from a porous matrix (size-exclusion chromatography). More recently, hydrophobic interaction chromatography, based upon bonding between nonpolar amino acids on protein surfaces with solid hydrophobic supports, also has been applied to wheat proteins (Popineau and Godon. 1978; Caldwell, 1979; Chung and Pomeranz, 1979). These separations, however, frequently do not adequately resolve complex mixtures and may not separate homologous proteins of similar size and charge. High-performance liquid chromatography (HPLC) overcomes these deficiencies by providing powerful new separation methods. Particularly relevant to proteins are the more
Fig. 5D-9. Allelic variation in high-molecular-weight (HMW) subunits of wheat glutenin' and their relationship to breadmaking quality. Standard HMW subunits of Chinese Spring coded by genes on chromosomes 1A, 1B, and 1D (the Glu-A1, Glu-B1, and Glu-D1 loci) are indicated on the left. Letters a–k designate each allelic group. Subunits more strongly associated with good quality are on the left. From Payne et al. (1984b).

porous (typically ≥300 Å) silica-based columns (Regnier and Gooding, 1980; Hearn et al., 1982; Wehr, 1984). The HPLC technology is now being applied extensively to wheat proteins; this section will briefly review the methods and their advantages, show some typical separations, and give examples of applications of HPLC to analysis of wheat gluten proteins.

5D-2.1 Reversed-Phase HPLC

High-resolution HPLC separations of cereal proteins were first obtained by Bietz (1983, 1985). A typical chromatogram of wheat gliadin is presented in Fig. 5D-10. Such a separation can easily be obtained, using any system with gradient capabilities that is equipped with one of the many available large-pore silica-based columns recommended for
Fig. 5D–10. Reversed-phase high-performance liquid chromatography (RP-HPLC) of gliadin proteins (cv. Centurk) extracted from a single wheat kernel with 70% ethanol and analyzed at 70 °C on a SynChropak RP-P (C18) column 4.1 × 250 mm, using a gradient from 28 to 30% acetonitrile in the presence of 0.1% trifluoroacetic acid during 55 min. and detection by absorbance (A) at 210 nm.

proteins. In reversed-phase (RP) HPLC, proteins interact hydrophobically with a nonpolar phase covalently attached to uniform porous silica. Initial RP-HPLC conditions are relatively hydrophilic, so that all proteins bind to the column; subsequently, the nonpolar nature of the solvent progressively increases, and proteins selectively elute in order of increasing hydrophobicity. The solvent system most useful for most proteins includes water, acetonitrile, and 0.1% trifluoroacetic acid; relatively nonpolar solvents may be especially useful for hydrophobic wheat proteins. Recent studies in our laboratory also have shown improved resolution at increased temperatures, perhaps because association of gluten proteins is minimized (Bietz and Cobb, 1985).

Excellent resolution is easily obtained by RP-HPLC for complex and heterogeneous mixtures. The total number of gliadins resolved (Fig 5D-10), for example, equals or exceeds resolution by most other methods, including two-dimensional electrophoresis. Electrophoretic analysis of single RP-HPLC peaks generally reveals highly purified proteins (Bietz, 1983; Burnouf and Bietz, 1984a; Huebner and Bietz, 1984); however, multiple components sometimes occur in single peaks, emphasizing wheat protein heterogeneity. Since RP-HPLC separates proteins by surface hydrophobicity, it complements chromatographic and electrophoretic procedures based on size or charge, thereby providing a powerful new tool for protein isolation, characterization, and comparison.

RP-HPLC has numerous other advantages. First of all, it is fast: optimal resolution requires about one hour with present technology, and considerably faster separations are possible. Run-to-run reproducibility is excellent; column-to-column reproducibility also is good, and columns are very stable. Good recovery of proteins from columns is achieved.
Proteins may be detected by absorbance of peptide bonds at 210 nm, making RP-HPLC extremely sensitive; half kernels can easily be analyzed, making the procedure nondestructive of germplasm. Alternatively, RP-HPLC is an excellent preparative method for gluten proteins (Bietz, 1983; Burnouf and Bietz, 1984a; Huebner and Bietz, 1984). The HPLC provides accurate quantitative data that are amenable to computer-assisted processing, replotting, and comparison. The RP-HPLC gives excellent separations of albumins and globulins (Bietz, 1983) and of glutenin subunits (Bietz, 1983; Burnouf and Bietz, 1984b) as well as of gliadin, making it suitable for all wheat polypeptides.

5D-2.1.1 Cultivar Identification

The RP-HPLC has already been used in numerous practical applications for wheat-protein analysis. One such application involves identification of wheat cultivars. Since gliadins are accurate genotypic indicators and since RP-HPLC resolves them at least as well as does one-dimensional electrophoresis, RP-HPLC can readily differentiate most wheat cultivars (Bietz, 1983). After optimal conditions for gliadin extraction for RP-HPLC were established (Bietz et al., 1984a), the method was applied to analysis of cultivars from all wheat classes (Fig. 5D–11) (Burnouf et al., 1983a; Bietz et al., 1984b). Most hard red spring wheats, as well as cultivars of other classes, could be readily differentiated by qualitative and quantitative differences among peaks. Cultivars with very similar pedigrees, such as Era and Solar, give similar patterns as observed by electrophoresis. However, because of improved resolution, complementary separations, and better quantitation, RP-HPLC may differentiate some cultivars that by electrophoresis appear identical. If chromosomal loci for gliadins are identical, RP-HPLC may still differentiate cultivars by their glutenin subunits (Burnouf and Bietz, 1984b) or albumin and globulin proteins. Thus, RP-HPLC can identify most wheat cultivars, and it may have certain advantages as compared to electrophoresis. For example, results for any sample are obtained in 1 to 2 h, rather than 1 to 2 days. Even though an operator can analyze fewer samples per day by RP-HPLC than by electrophoresis, recent studies (Bietz and Cobb, 1985), show that increased flow rates and temperatures, and rapid gradients, permit differentiation of most cultivars in only 10 to 15 min, with relatively minor loss of resolution.

RP-HPLC also is useful as a selection tool. For example, it can easily select atypical genotypes in 'Nap Hal' (Bietz et al., 1984b); this cultivar is a source of high-protein and high-lysine genes in breeding improved hard red winter wheat (Johnson et al., 1972). Similarly, genetic purity of cultivars may be established by RP-HPLC, and standard patterns can be defined for registration of genotypes.

5D-2.1.2 Prediction of Quality by RP-HPLC

As described above, numerous proteins related to wheat quality can be analyzed by electrophoresis. Because the resolution of RP-HPLC is
Fig. 5D-11. Differentiation of the hard red spring wheat cultivars (A) Butte, (B) Olaf, (C) Anza, (D) Era, and (E) Solar by reversed-phase high-performance liquid chromatography (RP-HPLC) of extracted gliadins. From Bietz et al. (1984b).
superior to that of many electrophoretic procedures, it also may reveal such relationships. This has already been confirmed in two separate instances, as follows.

Durum wheat gliadins give characteristic patterns by RP-HPLC, permitting cultivar identification and differentiation (Bietz et al., 1984b) (Fig. 5D-12). All cultivars examined had either peaks $b$ to $e$ (Fig. 5D-12A) or $i$ to $j$ (Fig. 5D-12C). This division of cultivars was identical to that based on electrophoretic bands 45 and 42, which predict gluten strength and weakness, respectively. Burnouf and Bietz (1984a) demonstrated that peak $b$ corresponds to band 42, and peak $i$ to band 45; they also used preparative RP-HPLC to isolate these proteins for further characterization and developed a rapid automated procedure (200 analyses per day) for screening durum quality during early stages of breeding.

High-MW glutenin subunits of hexaploid wheat related to bread-making quality (see previous discussion in this section) also may be analyzed by RP-HPLC. Using optimal conditions for reduction, alkylation, and RP-HPLC analysis of glutenin (Burnouf and Bietz, 1984b), high-MW subunits elute before those of lower MWs; differences among these subunits (Fig. 5D-13B, peaks $c$ and $e$) permit cultivar identification. Thus, since these peaks represent subunits that contribute in various degrees to bread-making quality, RP-HPLC can be used (as electrophoresis is used) to predict wheat quality.

RP-HPLC may become valuable for relating polypeptide compositions to technological quality in all types of wheat. Computer analysis of RP-HPLC data also may be useful for analysis of such factors and for identification of additional relationships of quality and protein.

5D-2.1.3 Use of RP-HPLC in Studies of Wheat Aneuploids

As noted above, electrophoresis, which permits locating genes that code specific polypeptides, is a valuable method for breeding and selection. RP-HPLC also can determine chromosomal control of proteins through aneuploid analysis (Bietz, 1983); for example, gliadins coded by genes on missing chromosome arms of Chinese Spring aneuploids can be easily identified, permitting their use as chromosome markers (see Fig. 6 of Bietz et al., 1984b). These studies, along with analyses of ‘Cheyenne’/Chinese Spring substitution lines and durum aneuploids, have been described elsewhere by Bietz and Burnouf (1985).

Locations of genes for glutenin subunits likewise can be demonstrated by RP-HPLC aneuploid analyses (Fig. 5D-14). For example, these results show that peaks $a$ and $b$ are coded by genes on chromosome 1D, and $c$ and $d$ by genes on the long arm of chromosome 1B. These peaks represent high-MW subunits correlated with quality (Fig. 5D-13), so their analysis by RP-HPLC may be valuable in breeding. A detailed study of glutenin subunit composition in Chinese Spring and in durum aneuploid lines has been completed, permitting assignment of most RP-HPLC peaks to specific chromosome arms (Burnouf and Bietz, 1985). In addition, RP-
HPLC has revealed significant differences among gliadins and glutenins of diploid, tetraploid, and hexaploid *Triticum* spp. and related genera, showing that the method is useful for the study of genetic and evolutionary relationships (Burnouf and Bietz, 1984c).
Fig. 5D-13. Variation related to breadmaking quality among high-molecular-weight glutenin subunits from the wheat cultivars (A) Marquis, (B) Hardi, and (C) Maris-Huntsman detected by reversed-phase high-performance liquid chromatography (RP-HPLC). From Burnouf and Bietz (1984b).
Fig. 5D-14. Reversed-phase high-performance liquid chromatography of pyridylethylated glutenin subunits from (A) the wheat cultivar Chinese Spring and from its aneuploid lines (B) nullisomic (N)D-tetrasomic (T)1A (N1DT1A), (C) N1BT1A, and (D) Ditelo 1B. From Bumouf and Bietz (1984b).
5D–2.2 Applications of Other HPLC Methods to Wheat Proteins

Although RP-HPLC has been the most successful HPLC technique applied to wheat proteins, size exclusion (SE) and ion exchange (IE) HPLC are also finding important applications. For example, resolution of wheat proteins by SE-HPLC (Bietz, 1984) is similar to that of traditional size-exclusion chromatography, but SE-HPLC has better reproducibility, accuracy, and ease of quantitation, and permits rapid (ca. 20 min) analyses in comparison to traditional methods, which may take days. SE-HPLC also may reveal differences in protein-MW distributions of wheat cultivars, which in turn may reveal bread-making quality.

Since ion-exchange chromatography is one of the best techniques for wheat protein fractionation, IE-HPLC should also be very useful (Bietz, 1985). Batey (1984) showed that resin-based “fast protein liquid chromatography” anion-exchange columns can identify wheat cultivars rapidly (in about 20 min) (Fig. 5D–15). Several other IE-HPLC columns tested, however, have not given good resolution of wheat endosperm proteins (J. Bietz, unpublished observations; I. Batey, CSIRO Wheat Research Unit, North Ryde, N.S.W., Australia, personal communication). It is probable, however, that a better understanding of how to use these columns, combined with improvements in columns themselves, will per-

Fig. 5D–15. Separation of gliadin proteins by anion-exchange fast protein liquid chromatography (Mono-Q column, pH 10.4, gradient 0–0.5 M sodium acetate) for identification of the wheat cultivars (a) Condor, (b) Durati, (c) Egret, and (d) Eagle. From Batey (1984).
mit high-resolution IE-HPLC separations of wheat proteins, and will lead to further improvements in all other HPLC methods.

5D-3 IMMUNOCHEMICAL APPROACHES

Protein relationships based on electrophoresis or chromatography are necessarily indirect, because unique polypeptides could have similar size, charge distribution, and hydrophobicity; therefore, additional, more specific approaches are desirable. This section will briefly review one such approach, the use of immunochemical procedures, and will show their value in assessing structural similarity of proteins, and analyzing species and genome relationships. Reviews dealing with this subject include Ewart, 1977; Konarev et al., 1979; and Daussant and Skakoun, 1983.

Immunochemical reactions depend on the interaction of proteins (antigens) with antibodies, which are immunoglobulins produced by the defense mechanism of higher vertebrates. Each antigen may possess several structural features, known as antigenic determinants, for which unique antibodies are produced. Antigens and antibodies frequently interact to form insoluble complexes; this interaction may occur as they diffuse through a gel matrix, forming insoluble precipitin lines, as in double diffusion, immunoelectrophoresis, rocket immunoelectrophoresis, and crossed immunoelectrophoresis (Daussant and Skakoun, 1983). Newer methods offer additional advantages. ELISA techniques (enzyme-linked immunosorbent assays) rely on binding of enzyme-coupled reagents to antibodies, so that antigen-antibody complexes are ultimately detected by a sensitive coupled enzymatic reaction, making ELISA much more sensitive than other immunochemical procedures. ELISA has been successfully applied to gliadin (Windemann et al., 1982). Alternatively, proteins that have unusual solubilities and are not amenable to standard immunological assays may be analyzed after immobilization on a nitrocellulose membrane (Dierks-Venting and Cozens, 1982) or chromatography paper (Esen et al., 1983).

Early immunological studies showed several gliadins to have common antigenic determinants, and suggested that gliadin and glutenin possess common structural features (Elton and Ewart, 1963; Grabar et al., 1965; Ewart, 1966; Beckwith and Heiner, 1966). These findings have been confirmed by sequence analysis (see below), but cross-contamination of gliadin and glutenin is possible in some studies.

Immunological comparisons of gliadins, albumins, and globulins have also been used to compare Triticum spp. and closely related genera. Proteins of the A, B, and D genomes of wheat have considerable structural resemblance (Ewart, 1966, 1969). However, some hexaploid wheat albumins characteristic of the D genome are absent in tetraploid wheat (Bozzini et al., 1970) and may reveal bread wheat in macaroni (Piazzì et al., 1972). Analysis of such proteins may best reveal genomic interrelationships among cereals (Konarev, 1981). Studies that examined antigenic
similarity of nongliadin proteins from *Triticum* spp. and closely related genera (Bozzini et al., 1970; Aniol, 1974; Konarev, 1981) confirmed knowledge of genome origins, and added some new knowledge concerning evolutionary relationships. However, in one study, using immunoelectrophoresis to assess phylogenetic relationships, enormous albumin/globulin variation was found within as well as between species (Rao, 1976). Limited cross-reaction has also been found between gluten proteins and similar prolamin fractions from fairly distantly related genera, such as *Hordeum* and *Zea* (Elton and Ewart, 1963; Kling, 1975; Dierks-Ventling and Cozens, 1982; Esen et al., 1983), indicating either significant sequence homology (as in ω-gliadin and C-hordein) or conservation of antigenic determinants representative of a common ancestor.

**5D-4 PROTEIN SEQUENCE ANALYSIS**

Determination of protein sequences is the ultimate technique for establishing genetic relationships. Electrophoresis and chromatography indicate only sequence similarity, since mutations may or may not lead to differences in charge, hydrophobicity, or size. This section briefly reviews the use of protein and nucleic acid sequence analysis to predict relationships and clarify origins of gliadin and glutenin polypeptides, and to determine evolutionary relationships among *Triticum* spp. and closely related genera.

Gliadin was first partially sequenced by Kasarda et al. (1974). Bietz et al. (1977) subsequently found two types of related gliadin sequences, as well as extensive homology within each group. One group consisted primarily of α- and β-gliadins, which are coded for by loci Gli-A2, Gli-B2, and Gli-D2 on the short arms of chromosomes 6A, 6B, and 6D, respectively (Payne et al., 1984b). Another group, the γ-gliadins, had different though still homologous sequences coded by gene loci Gli-A1, Gli-B1, and Gli-D1 on the short arms of chromosomes 1A, 1B, and 1D, respectively (Payne et al., 1984b). This homology suggests that gliadins originated through a process involving gene duplication, mutation, and polyploidy.

The complete sequences of three α-type gliadins have recently been determined from amino acid sequencing and from the sequences of cloned complementary DNA or genomic clones (Kasarda et al. 1984b, Anderson et al. 1984, Rafalski et al. 1984). Five sequence domains occur, two of which may have evolved from an ancestral gliadin gene. Significant homology occurs among these gliadins. In addition, Kasarda et al. (1984b) found evidence of homology between γ-3 and α-type gliadins.

Another group of gliadins, the ω-gliadins, have several variants of a significantly different sequence (Kasarda et al., 1983). These proteins, like γ-gliadins, are coded by the Gli-A1, Gli-B1, and Gli-D1 loci (Payne et al. 1984b), and ω-gliadins are homologous to γ-3 gliadin (Shewry et al. 1980,
Kasarda et al. 1984a). Thus, in spite of considerable divergence, all gliadin proteins seem to share a common ancestor.

Homology among gliadins coded at various loci has permitted several intra- and intergeneric comparisons. For example, ω-gliadin from T. monococcum is homologous to barley C-hordein (Shewry et al. 1980), to ω-secalin of rye, and to ω-gliadins of tetraploid and hexaploid Triticum spp. (Kasarda et al. 1983). Similarly, rye secalins of 40 000 and 75 000 MW are homologous to T. aestivum γ-2 and -3 gliadins (Shewry et al. 1982). In addition, the marked homology among prolamins within most cereals permits sequence analysis of heterogeneous mixtures and use of the results to predict and clarify evolutionary relationships among genera (Bietz 1982).

Information is also becoming available concerning sequences of wheat glutenin subunits. Bietz and Wall (1980) first determined partial N-terminal sequences for low-MW ethanol-soluble subunits, which were identical to subunits of high-MW gliadin. Although their sequences differ significantly from those of other gliadins, they are synthesized by genes at the same complex loci on the short arms of chromosomes 1A, 1B, and 1D (Gli-A1, Gli-B1, and Gli-D1) that code ω- and γ-gliadins (Payne et al., 1984b).

Sequence information for high-MW glutenin subunits, coded by genes on the long arms of chromosomes 1A, 1B, and 1D (the Glu-A1, Glu-B1, and Glu-D1 loci, respectively), is now also becoming available. Initial studies of these polypeptides revealed no free terminal amino groups (Bietz, Huebner, and Wall, unpublished observations); subsequently, this lack of reactivity was shown to result from acid-catalyzed cyclization of N-terminal glutaminyl residues to pyroglutamic acid, which is unreactive during Edman degradation (Shewry et al., 1984). In spite of these difficulties, Shewry et al. (1984) obtained partial sequence data for several high-MW glutenin subunits from T. aestivum, T. monococcum, and Ae. squarrosa (T. tauschii), which were shown to be homologous. DNA sequence analysis is now also rapidly revealing information about these subunits. Thompson et al. (1983) showed that high-MW glutenin subunits contain an internal 18-base pair repeat that codes the sequence -Gly-Gln-Gln-Pro-Gly-Gln--; similar results were obtained by Forde et al. (1984). These studies will soon reveal complete sequences for high-MW glutenin subunits, permitting better comparisons of proteins within and between genera, and finally offering the real possibility of relating protein structures to functionality.

5D-5 CONCLUSION

Several techniques, including electrophoresis, chromatography, immunoochemistry, and sequence analysis of endosperm proteins, can be used to better understand the biology, biochemistry, and genetics of wheat. These proteins are extremely heterogeneous and their expression is nearly
constant, making them excellent genotypic indicators. Consequently, protein analysis can identify genotypes, trace or establish evolutionary relationships, and determine the chromosomal control, interrelationships, and origin of proteins. Such knowledge may be applied to taxonomy, and protein markers of specific characteristics may be identified. In addition, protein analyses allow screening for good quality, and improved knowledge of structures is leading to a better understanding of the contribution of proteins to dough functionality. Further improvements in these methods, as well as new techniques, will permit better separation, characterization, and comparison of wheat proteins to predict, measure, improve, and assure the good quality of wheat, our most important food crop.
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