Computer-Aided Analysis of Gliadin Electrophoregrams. III. Characterization of the Heterogeneity in Gliadin Composition for a Population of 98 Common Wheats[1]

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ABSTRACT

The heterogeneity of gliadin composition resolved by polyacrylamide gel electrophoresis (PAGE) was studied to determine distinguishable groups of common components among a diverse collection of genotypes. The frequency distribution of protein bands, as a function of relative mobility, was computed. The resulting "PAGE map" confirmed the existence of at least 95 different gliadin components among a population of 98 common spring and winter wheat cultivars. For most cultivars, apparently common bands were identifiable as isolated clusters within a relative mobility interval of about 0.5 units. Rigorous standardization of electrophoretic mobility data, using multiple reference bands, was required to prevent significant gliadin heterogeneity from escaping detection. The PAGE map process is proposed as a reference method for analysis of one-dimensional electrophoretic data facilitating an objective classification of protein composition for multivariate or genetic analysis and interlaboratory comparisons.

The heterogeneity of gliadin components and their differing resolution and identity by different electrophoretic and nomenclature systems (reviewed by Wrigley et al 1982a), limit the utility of results for broad-based comparisons. Furthermore, a more fundamental problem is that, even with the same electrophoretic procedure, direct identification of apparently common components among patterns of different genotypes is rarely obvious. Therefore, considerable simplification and possibly erroneous data may result from subjective interpretation of electrophoregrams or derived relative mobilities used to classify band patterns of individual genotypes. This process is crucial to study of protein composition for genetic relationships (e.g., Sozinov and Poperelya 1980) or statistical association with utilization quality parameters (Wrigley et al 1981).

In view of the multiplicity of gliadin components (Kasarda et al 1976), their close proximity in the electrophoregrams, and the confounding effects of experimental variability, a subjective approach to formulating a classification of gliadin composition is not practical. Moreover, gains in PAGE resolution that improve its discrimination power for cultivar identification have made band classification more difficult.

We reported (Sapirstein and Bushuk 1985a) a new relative mobility (Rm) standardization procedure that uses three reference bands to improve precision of results. An important feature of this procedure is the improved ability to detect small differences between bands. The present article describes the classification of gliadin bands from Rm data derived by the three-band standardization method.

MATERIALS AND METHODS

Wheat Samples and Electrophoresis System

Grain samples of 98 common spring and winter wheat cultivars were supplied by the Research Branch of Agriculture Canada and R. Tkachuk of the Grain Research Laboratory, Winnipeg. This material comprised a diverse collection of hard and soft wheat genotypes, largely of Canadian origin, developed and licensed for commercial production over 90 years. A listing of cultivar names and electrophoregram formulas is presented in Figure 1. A classification of material according to kernel type has been previously given (Sapirstein and Bushuk 1985b).

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Extraction of gliadin from meal of bulk grain and single kernels with 70% ethanol, and electrophoresis in 6% polyacrylamide gels was carried out as described by Sapirstein and Bushuk (1985a). An average of three replicate electrophoregrams were prepared and analyzed for each cultivar. Different electrophoregrams were obtained for two samples from different sources of cultivars Preston and Jr. No. 6. Samples designated Preston-A and Jr. No. 6-A in Figure 1 are considered to be authentic, based on pedigree information and/or kernel characteristics.

Quantification of Electrophoregrams and Computer Analysis

Methods for determining relative mobility and band intensity values and coding data, as electrophoregram formulas, for computer analysis have been described (Sapirstein and Bushuk 1985a,b). The relative intensities of bands were quantified by being given an integer value in the range of 1 (very faint) to 9 (very dense) based on visual evaluation of electrophoregrams. A Talos model 648 digitizing tablet was used to determine band migration distances from photographic prints of electrophoregrams. Data were transferred to an Amdahl 580 mainframe computer for analysis.

Absolute band position data were normalized to relative mobilities using the three-reference band standardization procedure of Sapirstein and Bushuk (1985a) and the single reference band procedure of Bushuk and Zillman (1978). A FORTRAN computer program, designated "STATWT3," was used to make the calculations. Another FORTRAN program ("FORMULA"), using CALCOMP (California Computer Products, Anaheim, CA) subroutines, generated a plot of electrophoregram formulas tabulated under a single mobility scale for cultivar comparisons (as illustrated in Fig. 1). Program "PAGEMAP" was developed to produce a composite diagram from a population of input cultivar formulas. The output, herein termed a "PAGE map," represents a frequency histogram of bands as a function of Rm. To minimize occurrence of overlapping distributions, the mobility scale was divided into 800 (0.1 Rm unit) segments from 10.0 to 90.0, covering the full range of experimental values. Each data point represents the relative intensity of a band at a specific Rm. PAGE map resolution is in accord with uncertainty of relative mobilities: standard errors reported previously (Sapirstein and Bushuk 1985a) are less than ±0.08 Rm units. Annotated listings of the computer programs used in this study can be obtained from the first author on request.

RESULTS AND DISCUSSION

Typically, 30–40 gliadin bands were resolved in each of the 98 replicated sets of electrophoregrams examined (Fig. 1). This resolution is comparable to that recently obtained by one-dimensional PAGE (Lafiandra and Kasarda 1985) but higher than that obtained in earlier PAGE studies (e.g., Jones et al 1982, Zillman and Bushuk 1979) because of improved resolution of the present electrophoretic system, the inclusion of very faint bands (density = 1), and the interpretation of partially overlapping bands (having a well-defined leading or trailing shoulder) as two closely contiguous components. Analysis of the average of three replicate PAGE patterns per cultivar minimized the subjectivity in band identification. Only reproducible components were retained.

Three Reference Band PAGE Map

Figure 2 is a composite pattern (PAGE map) of the electrophoregram data listed in Figure 1. An enlarged segment of Figure 2 is shown in Figure 3, illustrating the typical underlying distribution of band densities which exists in the data. The most prominent feature of this result is that gliadin heterogeneity is sufficiently discontinuous so that bands of similar mobility are generally identifiable as isolated clusters of points in the PAGE map. Overall, approximately 90 such clusters may be counted. Fewer than eight comprised infrequent components which are present in less than 15% of the electrophoregrams that were studied (Rm clusters 12.6–12.8, 23.3–23.5, 34.8–36.2, 51.0–51.3, 66.4–66.7, 66.9–67.1). Two clusters are essentially invariant bands characterized by mobilities in the range 45.6–46.2 and 59.0–59.8. The first cluster of relatively dense bands, according to evidence provided by Bushuk and Zillman (1978), likely corresponds to "band 65" as designated by Autran and Bourdet (1975).

Figure 2 also shows a limited number of "classes" comprising only one or two components (e.g., bands with Rm: 25.0, 25.7, 30.8, 44.1, 78.3, and 80.3). The mobilities of these proteins have been verified by replication. No doubt a small number of gliadins are poorly represented in the present collection of cultivars; however, the presence of novel or altered protein bands arising from mutational events cannot be excluded as well.

The unimodal and generally symmetrical band distributions in Figure 2, typically spread over 0.4–0.5 distance units, indicate that many are apparently homogeneous, although numerous instances of incomplete resolution were observed. For example, the bimodal distribution between Rm 26.2 to 26.9 indicates the presence of at least two distinct gliadin bands in this region. Slightly overlapping gliadin band clusters are also evident in the Rm ranges 40.8–41.7 and 75.7–76.5, especially if differences in band intensity are taken into account.

Severely overlapping band distributions were observed within mobility ranges 58.0–58.7 and 61.3–62.4. The second of these, which contains a broad cluster of 107 points, is plainly heterogeneous because at least nine bands of the 98 cultivars are represented twice. Given the spread of bands over 1.2 Rm units, and the shape of the cluster showing prominent leading and trailing perturbations, it is likely that at least three distinct populations of bands are present in this poorly resolved mobility region. The skewness of some unimodal clusters (e.g., centered on mobilities 27.7, 42.8, 44.7, 47.7, 48.7, 71.5, and 81.5) may also indicate a complex composition.

In general, overlapping bands that were resolved before computer analysis, and poorly resolved clusters mentioned above, were most prevalent in the intermediate mobility region corresponding to β- or γ-gliadins. This result is consistent with what is known about the genetic determination of the gliadins, as twice the number of chromosomal loci, code for these proteins compared to other individual gliadin groups (see Payne et al 1984, for review).

Clearly, for the purpose of band classification, some difficulty exists in the separation of variate populations which overlap considerably, thus reflecting the limitations which exist for any one-dimensional electrophoretic technique. A possible improvement for the present methodology might be to add reference bands in the relevant mobility regions. This would represent only a relatively minor programming task (Sapirstein and Bushuk 1985a). The benefit would be twofold: 1) a significant narrowing of the Rm distribution for the reference band population to approximately 0.2 distance units as indicated in Figure 1 for bands R24, R50, and R79; and 2) a reduction in the error component for neighboring bands. It has been shown (Sapirstein and Bushuk 1985a) that the uncertainty in band Rm approaches a minimum in the vicinity of the reference band position. The combination would therefore result in an improved separation of the various overlapping gliadin band distributions. In fact, conceivably every major band in the standard protein patterns (i.e., cultivars Marquis or Neepawa) used to normalize band migrations distances could be employed as reference markers, so that virtually any dectectable difference between bands could be interpreted as arising from heritable factors.

Single Reference Band PAGE Map

The effectiveness of the three reference band normalization procedure on the characterization of gliadin electrophoregram heterogeneity can be demonstrated in a straightforward way: we generated a composite pattern, from the identical set of migration distance data, normalized to relative mobilities using a single reference band approach (Bushuk and Zillman 1978). The resulting PAGE map (Fig. 4) shows a substantial decrease in the number of differentiated band populations compared with Figure 1. Differences were largely localized in the low- and high-mobility regions where the two standardization methods differ in reference

band placements. Thus, although the resolution of band classes in Figure 4 with mobilities below Rm 35 appears to be satisfactory, the data are substantially more scattered than Figure 2, which reveals that a much higher resolution exists.

Perhaps the most striking feature of Figure 4 is the continuous nature of the PAGE map above Rm 70, where all distinctions between gliadin band clusters are obscured. This result is consistent with the relatively high level of variability in Rm values for high-mobility bands using the single reference band approach (Sapirstein and Bushuk 1985a). It should be noted as well that the magnitude of standard error associated with these bands (±0.4 Rm units), is comparable to the average variability generally reported in the literature (Lookhart et al 1983, Zillman and Bushuk 1979). This suggests that attempting to classify gliadin bands either visually or by their mobility values, given the typical level of reproducibility obtainable by traditional standardization methods, is a very uncertain process.

General Discussion

Our results indicate that precision-related factors can substantially undermine the interpretation of electrophoregrams, even from numerical data. A parallel result was obtained by Mansur-Vergara et al (1984) in a recent study of genetic variability in *Triticum dicoccoides*. These workers showed that except for

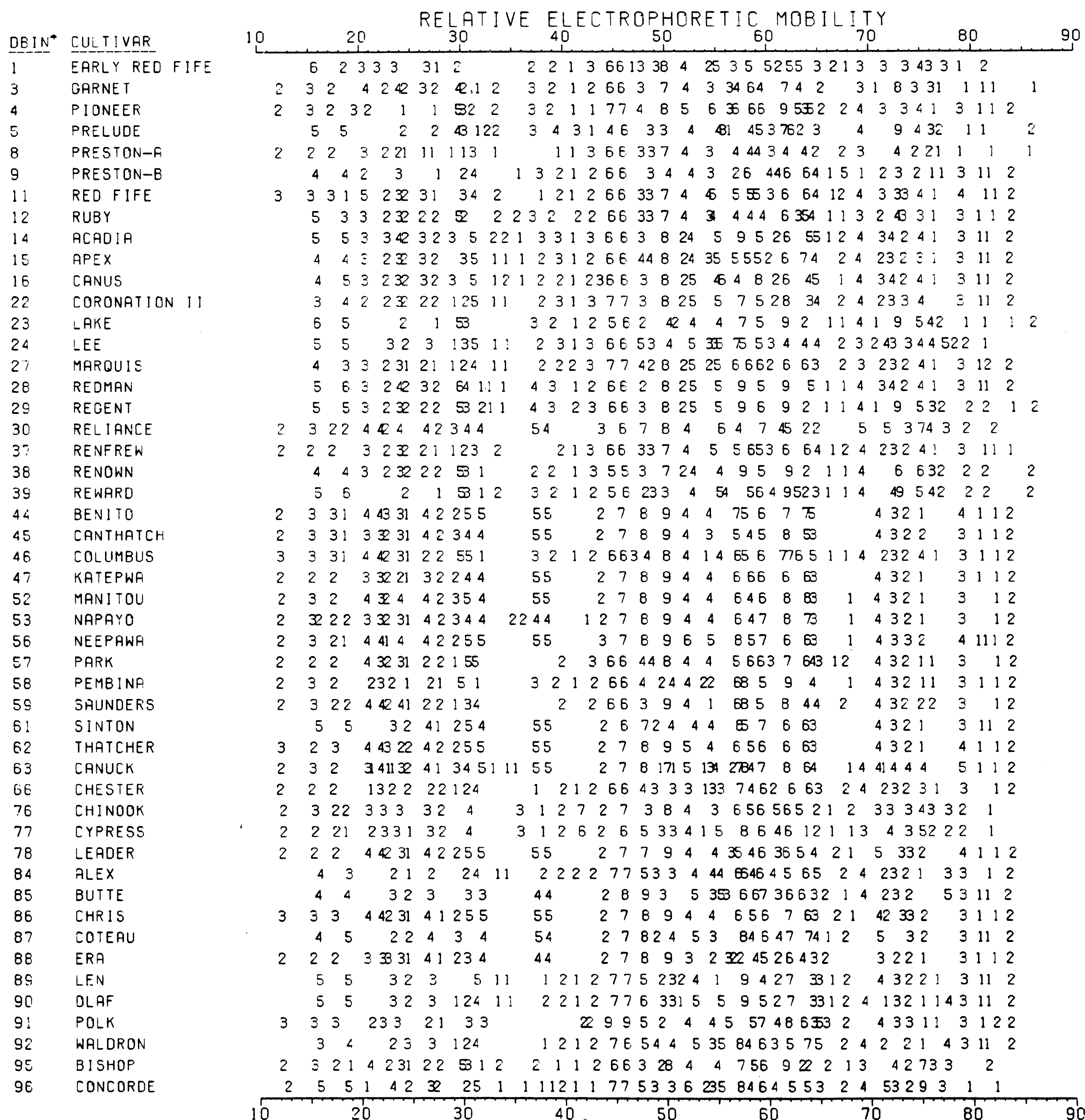

Fig. 1. Gliadin electrophoregram formulas of common wheat cultivars. The position of each band in the mobility field is indicated by its relative intensity value. DBIN = Data base identification number. Corresponding list of cultivars by grain class/type is provided in Sapirstein and Bushuk (1985b).

proteins of high molecular weight, sodium dodecyl sulfate (SDS)-PAGE of total protein extracts may classify constituent polypeptides in a computer-generated composite pattern of subunits. It was speculated that the appreciable uncertainty in high molecular weight bands (i.e., glutenin) in SDS-PAGE contributed to an apparent absence of distinct clusters in the high molecular weight region.

From the foregoing, it is evident that rigorous standardization of band migration distances is required to prevent significant levels of protein heterogeneity from escaping detection by electrophoresis. Thus more than 95 different gliadin proteins can be readily identified from the PAGE map in Figure 2. By comparison, a substantially lower heterogeneity has been characterized elsewhere, in studies requiring a classification of gliadins to determine significant associations between electrophoregrams (Branlard 1983) or genotypes and relationships to end-use quality (Branlard and Rousset 1980, Branlard and Bellot 1983, Wrigley et al 1981, 1982b,c).

Branlard and Rousset (1980), for example, partitioned starch gel electrophoregrams from 50 cultivars grown in France into 43 Rm classes, using the single reference band gliadin nomenclature of Autran and Bourdet (1975). The band classification of Wrigley et al (1981), also by starch gel electrophoregrams, involved a total of 34 gliadin band classes among 80 genotypes of mainly Australian origin. Bands were assigned to numbered classes based on visual interpretation of electrophoregrams. Branlard and Bellot (1983)

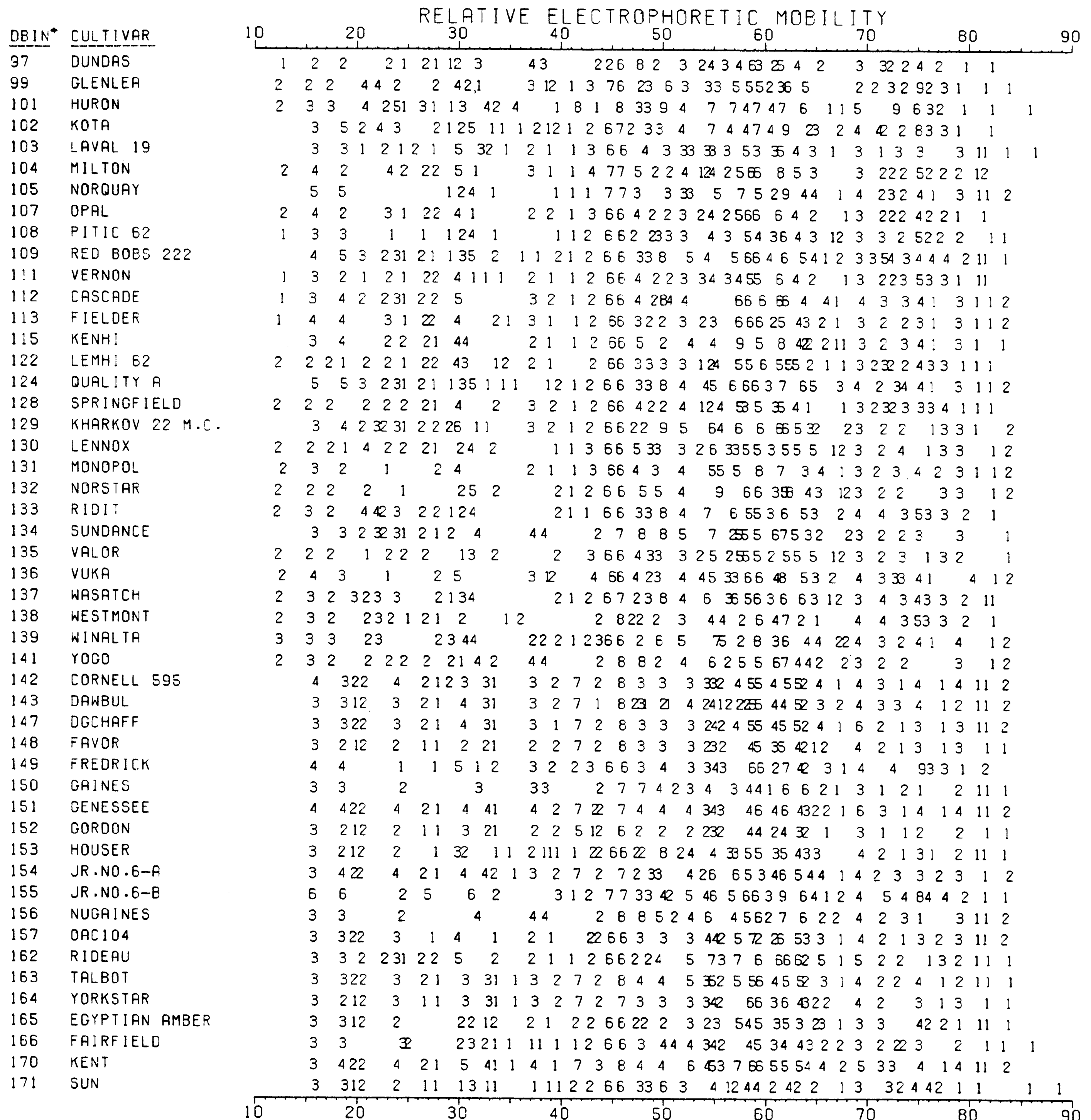

Fig. 1. Continued from previous page.

assigned a total of 55 gliadins to different mobility classes by PAGE analysis of 61 cultivars. These studies have provided considerable evidence of a significant relationship between numerous specific gliadin components and quality attributes for French and Australian wheats. However, none discussed the repeatability in identifying named classes and allocating bands to them.

Similar considerations also apply to the extensive genetic nomenclature of gliadins which has been developed (Sozinov and

RELATIVE ELECTROPHORETIC MOBILITY

Fig. 2. Three reference band PAGE map of gliadin composition for 98 wheat cultivars. The computer-generated diagram is a composite of electrophoregram data listed in Figure 1. Relative mobilities were determined by the three reference band method of Sapirstein and Bushuk (1985a). Peaks designated R24, R50, and R79 specify reference band positions. Plotted numbers are band relative intensities. Figure size is reduced to 20% of the original computer output, which presents results on a continuous mobility axis.

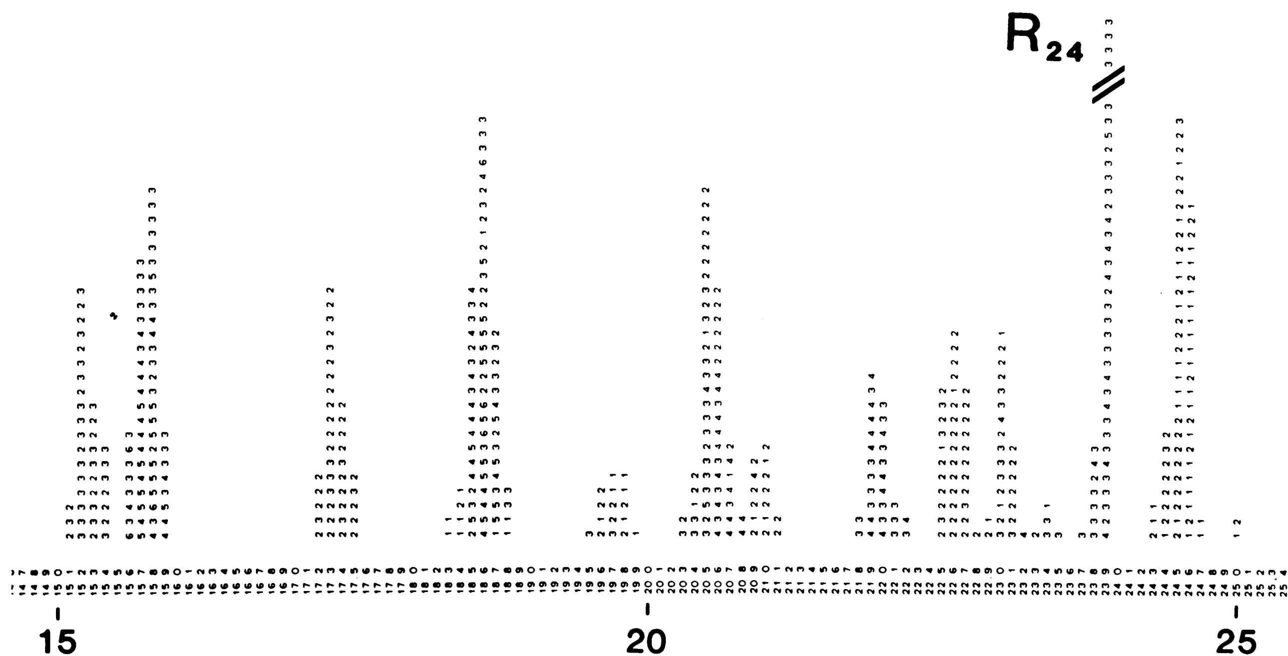

Fig. 3. Section of PAGE map in Figure 2 representing at least 14 different gliadin components within an interval of 10 Rm units. The illustration is reproduced to 60% of original size to reveal distribution of band densities.

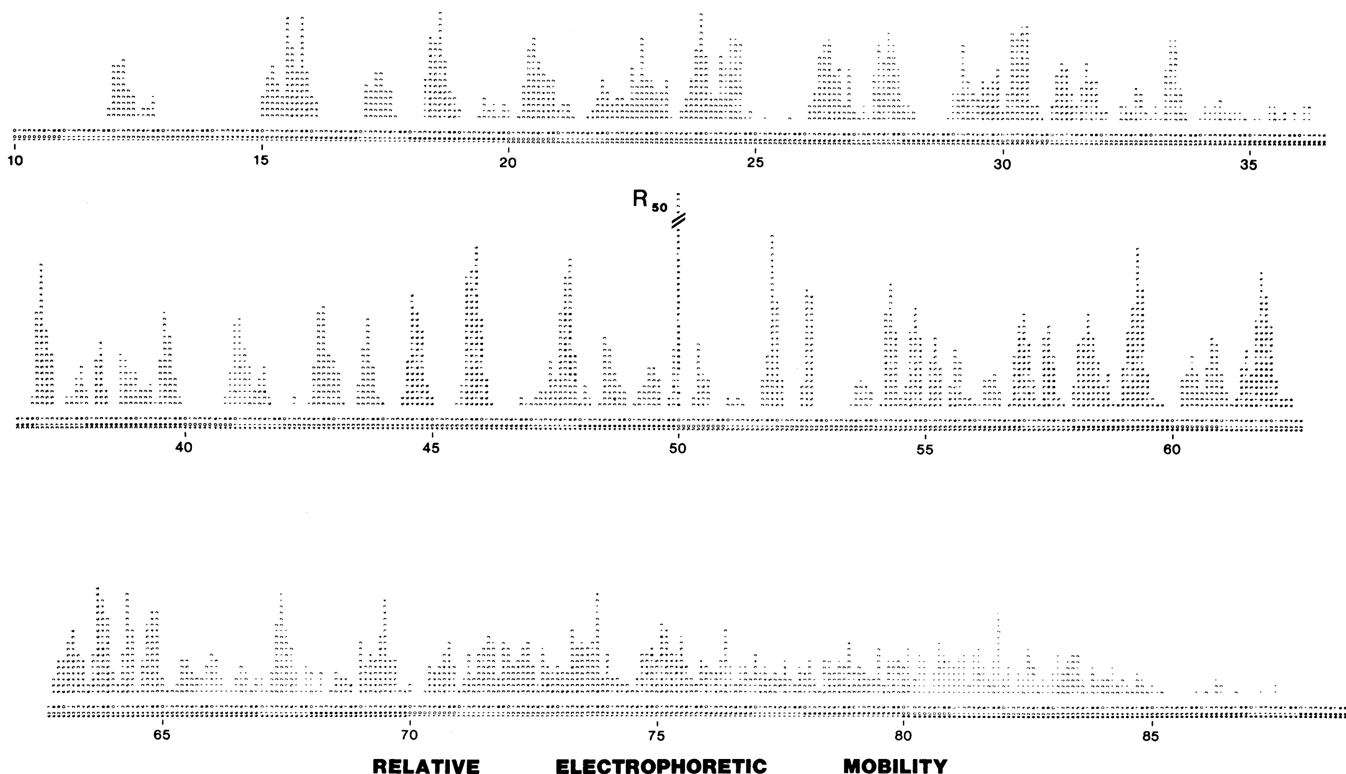

Fig. 4. Single reference band PAGE map of gliadin composition showing analogous result to Fig. 1 but with band migration distances normalized to relative mobility scale using the single reference band method of Bushuk and Zillman (1978).

Poperelya 1980, Metakovsky et al 1984) designating allelic blocks or subpatterns of tightly linked bands and their assignment to genes on group-1 and group-6 chromosomes that control their synthesis (Shepherd 1968, Wrigley and Shepherd 1973). More than 125 distinct gliadins may be counted in the catalog of blocks (Metakovsky et al 1984) specifying protein components coded by six complex loci. As specific gliadin blocks have also been related to grain quality (Sozinov 1984), the potential of this approach in plant breeding will only be fully utilized if a suitable reference pattern is available. Such a reference pattern should contain a standardized numerical scale to which sample electrophoregrams may be compared in a relatively unambiguous fashion.

Our standardization methodology appears to be well suited for these applications, as it provides an objective basis for quick and detailed comparative analysis of many samples. The inclusion of a standard set of well-documented and diverse genotypes (e.g., Besostaya 1, Cheyenne, Chinese Spring, Marquis) into the PAGE map should further improve its utility for broad-based comparisons. As the computed result provides an objective estimate of gliadin heterogeneity for a given set of wheats, this will be helpful in future studies on the association between protein quality or taxonomic relationships and electrophoregram composition. The PAGE map methodology, incorporating multiple reference bands for Rm determination, should clearly be advantageous in these types of multivariate analysis research by increasing the number of reproducible Rm classes. This will consequently diminish the undesirable effects of combining data from truly different proteins that may not be obvious from direct inspection of either electrophoregrams or mobility valves.

Still, the fact remains that we may have characterized only about one-half of the total heterogeneity of gliadin composition, as two-dimensional electrophoretic techniques can typically double the number of components resolved by one-dimensional PAGE (Wrigley and Shepherd 1973). Nevertheless, the advantage of our methodology relates to the ease with which discriminant mobility boundaries and the classification of proteins may be established from complex PAGE data when band scatter due to experimental error is minimized. This approach may also provide a more precise basis for comparison of two-dimensional PAGE results than has been previously described (Lafiandra et al 1984).

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