Computer-Aided Analysis of Gliadin Electrophoregrams. I. Improvement of Precision of Relative Mobility Determination by Using a Three Reference Band Standardization[1]

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ABSTRACT

Gliadin electrophoregrams used to identify wheat cultivars were analyzed by a computer-aided procedure. The reproducibility of relative mobility (Rm) determination using a standardized electrophoretic technique in 6% polyacrylamide gels was evaluated over a five-month period. Migration distances of bands were measured from photographs of electrophoregrams using a digitizing tablet that fed the values directly to a computer for processing. Absolute positional data was normalized to an Rm scale using the single reference band 50 nomenclature of Bushuk and Zillman (1978)

and a new three reference band system. The variation in Rm increased with increasing distance between a particular band and the reference band(s). Compared to the single reference band procedure, the use of three reference bands significantly improved precision of results by stabilizing the variability in Rm measurements at a low level over the entire electrophoregram. Mean standard errors below 0.08 Rm units were achieved with minimal replication, effectively increasing the discriminative power of electrophoresis for comparative analysis.

Electrophoretic separations of gliadin proteins are the most widely accepted laboratory techniques for differentiation and identification of wheat cultivars. Of the various methods available (Wrigley et al 1982), advantages of a uniform polyacrylamide gel system (Bushuk and Zillman 1978) have been recognized (Autran et al 1979, Redman et al 1980), and many experimental modifications have since been described (Cooke 1984). For our studies using a computer to compare gliadin electrophoregrams, it was crucial to minimize experimental error in band mobility. Based on relative mobility (Rm) standardizing nomenclatures using single reference bands in starch (Autran 1973, Autran and Bourdet 1975) and polyacrylamide gels (Bushuk and Zillman 1978), several catalogs of cultivar band mobility-intensity data arrays (electrophoregram formulas) have been reported for identification of wheats grown in different countries (Autran and Bourdet 1975, Dal Belin Peruffo et al 1981, Ellis and Beminster 1977, Jones et al 1982, Zillman and Bushuk 1979). Except for the first such catalog using polyacrylamide gel electrophoresis (PAGE) (Zillman and Bushuk 1979), relative mobilities for gliadin bands have been specified with integer precision. Zillman and Bushuk (1979), using a custom-built electrophoretic apparatus, found Rm precision for three selected Marquis bands to be within ± 0.5 units. Lookhart et al (1983), using a commercial apparatus, also found precision of Rm generally to be ± 0.5 units, but variations of ± 1 unit would sometimes occur, especially for bands with high mobilities.

No reports have systematically evaluated reproducibility of Rm determination for a complete spectrum of electrophoregram components, however, nor determined how long-term variability is affected. Furthermore, studies have not yet demonstrated that Rm precision is sufficient to reliably permit computer-based cultivar identification. These questions were investigated in an attempt to develop a computerized system to permit cultivar identification by gliadin electrophoregrams and to study gliadin heterogeneity. Results will be discussed in a series of articles. This initial article describes a new electrophoregram nomenclature, based on three reference bands, which has been adopted to improve precision of Rm determination and to provide more accurate wheat cultivar formulas which encode electrophoregrams.

MATERIALS AND METHODS

Wheat Cultivars

The cultivar Marquis was used as the primary PAGE reference

sample (Bushuk and Zillman 1978), and Neepawa was used as a supplementary reference because of its predominance in the Canadian hard red spring wheat class. Grain samples were obtained from the Plant Gene Resources of Canada, Ottawa Research Station, Agriculture Canada.

Chemicals and Reagents

All chemicals used were of reagent grade or better. Distilled deionized water was used to prepare gliadin extraction and electrophoresis solutions. Acrylamide, ascorbic acid, ferrous sulfate, and lactic acid were obtained from Fisher Scientific (Winnipeg, MB). Methylene-bis-acrylamide was obtained from both Fisher Scientific and Sigma Chemicals (St. Louis, MO). Aluminum lactate was obtained from Fluka Chemicals (Hauppauge, NY). Hydrogen peroxide (3%, practical grade) was purchased at a local pharmacy.

Gliadin Extraction

Single kernels were pulverized between halves of a folded sheet of weighing paper with a hammer. The meal was then transferred to a 1.5-ml microcentrifuge tube and extracted with 70% ethanol (2 μl/mg grain). After brief agitation on a vortex mixer, the mixture was left to stand at room temperature for 30 min, vortexed again, and contents were centrifuged for 5 min at 8,000 × g at room temperature using a tabletop microcentrifuge. The 30-min extraction time was used as a matter of convenience, as a 5-min extraction revealed no detectable difference in results. When bulk wheatmeal samples were extracted, a 50-mg subsample of ground grain (Udy cyclone mill) was substituted for the meal of a single kernel in the extraction procedure.

After centrifugation, an aliquot of the supernatant (e.g., 30 μl) was diluted with 1.3 times its volume of gliadin extract dilution solution and stored in a sealed vial. The extract dilution solution consisted of electrophoresis buffer (see below) containing 40% (w/v) sucrose to increase the density of the extract, and 0.6% (w/v) methyl green dye, which served as a marker of the progress of electrophoresis.

Electrophoresis

Polyacrylamide electrophoresis in 6% gels was performed as described by Bushuk and Zillman (1978), with a few modifications. The original horizontal apparatus was modified into a vertical version. This apparatus used 100 ml of gel solution to form a slab (20 × 15 cm) approximately 3 mm thick, giving superior gliadin resolution (Sapirstein 1984). Typical results are presented in the following section.

Recipes for gel solution and tank buffer are given in Table I. The concentration of ferrous sulfate was reduced by 36% compared to that used by Bushuk and Zillman (1978), significantly improving gel firmness, facilitating ease of handling, and improving reproducibility. Similar effects are described by Khan et al (1985).

After polymerization, the apparatus was oriented vertically and tank reservoirs were filled with 580 ml of buffer. Sample extracts (8 μl) were applied (11/gel slab) with a micropipette. The outside and center slots of the gel slab were used for reference gliadins (Marquis and/or Neepawa). Along with the center reference sample, each outlying reference sample was subsequently used to determine Rm values for electrophoregrams in their respective side of the gel slab. Compared to the method reported by Bushuk and Zillman (1978), this procedure increases the ratio of reference to test samples, thus minimizing effects of intragel variability on precision.

During electrophoresis, temperature was controlled by circulating water at 21°C. Duration of electrophoresis was approximately 4 hr, with a constant current of 60 mA (about 360 V [17 V/cm] across the gel). Runs were terminated when the tracking dye band with lower mobility migrated to about 1 cm from the opposite end of the gel. As observed by Khan et al (1983), this fixed-distance approach gave an optimal and uniform separation of bands.

Gel Staining and Photography

Gels were stained overnight in a filtered solution containing 0.1 g of Coomassie Brilliant Blue R-250 (dissolved in 10 ml of 95% ethanol) in 240 ml of 12% trichloroacetic acid (TCA). Gels were destained for 6 hr in 12% TCA. Cotton swabs were periodically used to remove any precipitated stain that adhered to top and bottom surfaces of the gel slab.

Gels were photographed using Kodak Technical Pan 2415 film to produce prints and positive (4 × 5 in.) transparencies. HC110 developer was used at medium dilution (9:1). Prints (8 × 10 in.) were used to determine band migration distances (see below). The film and development conditions used were found to give optimal contrast with a satisfactory grey level representation of electrophoregram results.

Computer Acquisition of Gliadin Band Migration Distances

Band pattern migration distances were acquired from photographic prints of gel slabs in a semiautomatic fashion, using a digitizing tablet to measure and input values directly into a computer file, thus obviating the need to manually transcribe data. Using the digitizer to quantify band migration distances substantially minimized the tedium, reading, and transcription errors inherent in manual measurement of band positions.

Each print was taped securely to a Talos 648 digitizing tablet having a resolution of 1,000 lines per inch at an accuracy of ± 0.005 in. For each electrophoregram, an initializing routine first defined coordinates for the origin. A hand-held cursor, having electronically wired crosshairs enclosed between two circular glass lenses, was placed over the leading edge of the center of the slot and a cursor key was depressed to digitize the point. Subsequently, each band in the electrophoregram was visually identified, and its migration distance (from the origin to band center) was digitized. In addition to sample band migration distances, for each electrophoregram, the equivalent positions of three reference bands were also determined. These were used to standardize electrophoregrams to facilitate precise comparisons of protein-pattern data from different electrophoretic runs.

TABLE I
Recipes for Gel and Tank Buffer Solutions

	Amount Required for 100 ml Final Volume
Gel solution	
Acrylamide	6.0 g
N,N'-methylene-bis-acrylamide	0.3 g
Ascorbic acid	0.1 g
Ferrous sulfate heptahydrate	0.0016 g
Catalyst solution	
Hydrogen peroxide, 3%	0.5 ml
Tank buffer solution	
Aluminum lactate	0.25 g
Lactic acid	to pH 3.1

RESULTS

Application of Three Reference Bands to Compute Relative Mobilities

To improve day-to-day reproducibility of Rm for comparative analysis of gliadin electrophoregrams and increase overall data base accuracy, a new three reference band system was developed. This system substantially modifies the single reference band method described by Bushuk and Zillman (1978). The two additional reference bands were designated R24 and R79 based upon their mobilities relative to that of the primary reference band R50 in electrophoregrams of Marquis and Neepawa (Fig. 1). Either or both cultivars were used to provide standard reference electrophoregrams for every PAGE run.

For each sample electrophoregram, reference band migration distances were determined, as described above, using their respective equivalent positions in the pattern. These were specified by lines drawn across the gel slab joining common reference bands in the outside and center standard electrophoretic patterns (Fig. 2). Because the density of band R24 is significantly higher in the Neepawa electrophoregram, this cultivar was the preferred control.
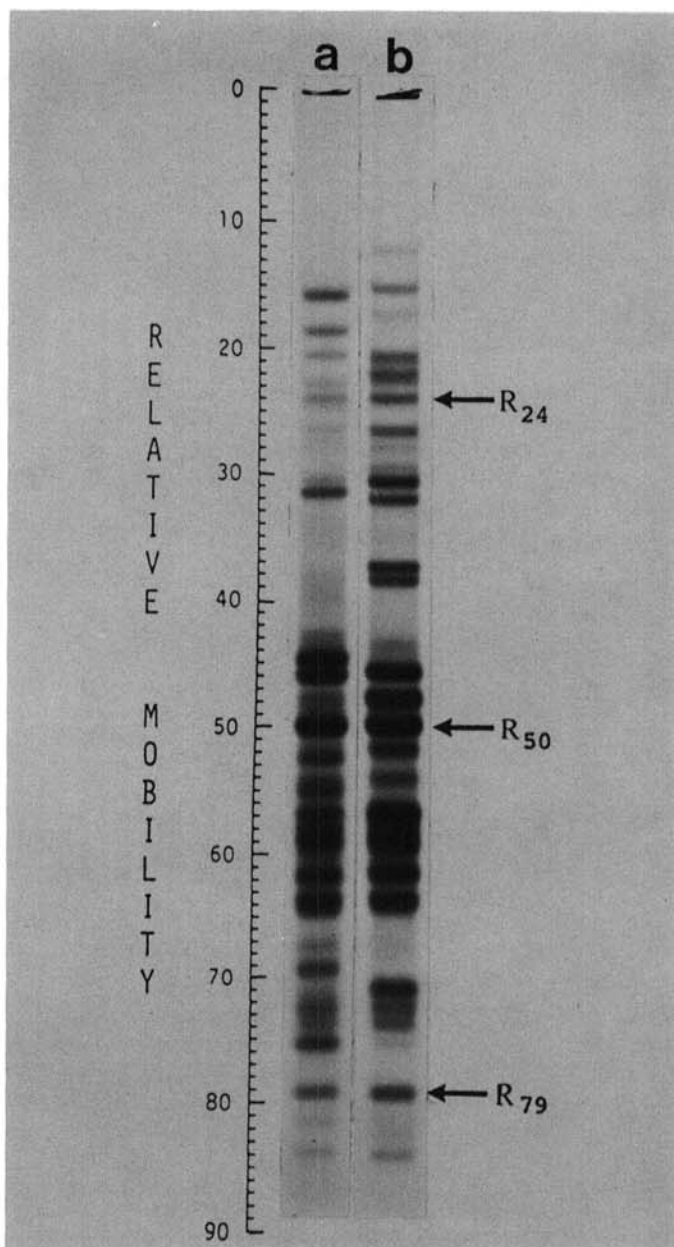

Fig. 1. Reference bands R24, R50, and R79 in gliadin electrophoregrams of cultivars Marquis (a) and Neepawa (b).

Its suitability as a new standard cultivar is also recommended on the basis of genetic homogeneity: no offtype patterns were found in PAGE analysis of over 150 single Neepawa kernels.

Incorporating reference bands R24 and R79 into the algorithm used to normalize migration distance data required accurate determination of their relative mobilities, following the method of Bushuk and Zillman (1978). The aim was to achieve a precisely linear relationship between migration distance and computed Rm for the complete range of experimental values. As indicated by electrophoregram results (Figs. 1 and 2), no significant difference was found in mobilities of corresponding reference bands in Marquis and Neepawa. On the basis of over 100 replicate determinations, gliadin reference bands R24 and R79 were assigned average values of 23.88 and 78.95 Rm units, respectively. As electrophoresis apparatus design is a factor that can significantly modify band relative mobilities (Autran et al 1979, Sapirstein 1984), different values may be obtained by other workers.

The relationships used to compute relative mobilities using multiple reference bands are given in Table II. The expression invoked to make the calculation (equation 1, 3, 6, or 7) is contingent upon the position of a gliadin band in the electrophoregram field. For example, if a gliadin component does not migrate as far as band R24, its mobility is determined relative to band R24 alone (equation 1). For proteins that migrate faster than R79, Rm is determined using the high mobility reference (equation 3). If a band

lies in the zone between two reference bands, its relative mobility is the sum of the function of both markers weighted according to the separation distances involved (equation 6 or equation 7).

Within their respective domains, the weighting functions $l_i(x)$ and $h_i(x)$ (Table II, equations 4 and 5, respectively), can range between 0 and 1 and are equal to 0 or 1 at the reference protein boundaries. Thus, as the mobility of a gliadin component nears that of a reference band, the contribution of the more distant counterpart to the computed relative mobility declines. For example, if a gliadin band approaches the mobility of reference band R50 from a higher or lower mobility, equations 6 and 7 will gradually assume the same identity; at $R_i(x) = 50.0$, the contributions of $Q_i(x)$ and $S_i(x)$ disappear. Similarly, the pair of equations 1 and 6 and equations 3 and 7 converge in identity for gliadin bands with mobilities approaching the low- and high-mobility reference proteins, respectively.

The task of standardizing band migration distances to relative mobilities was carried out by means of a computer program written in FORTRAN and designated "STATWT3." Arithmetic mean and standard deviation are generated as program output for each gliadin band along with the cultivar formula for the electrophoregram which includes assigned band densities on a 1–9 integer scale (Sapirstein and Bushuk 1985a). Because these algorithms are relatively simple and fast, program implementation using resources of a small microcomputer (64K memory) should be satisfactory. An annotated source listing of the computer program can be obtained from the senior author on request.

Precision of Relative Mobility Determination

A comparison of single and triple reference band standardization methods on the uncertainty of gliadin band relative mobility determination is presented in Figure 3. Statistical analysis of the two Rm measurement procedures was based on a common set of 13 replicate PAGE patterns, selected at random, of different gliadin extracts of Neepawa run on different gels over about five months. This time frame provided a suitable basis to evaluate the practical range of experimental variation as it affected the reproducibility of relative electrophoretic mobility.
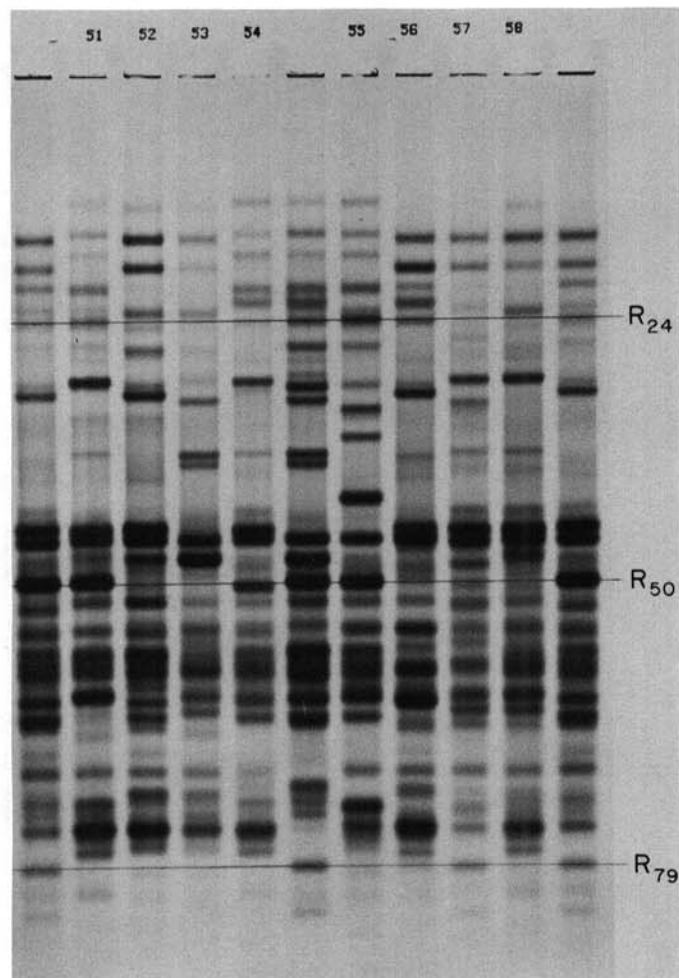

Fig. 2. Typical layout of electrophoregrams in photograph of gel slab prepared for digitization. Lines drawn across the figure specify in numbered sample electrophoregrams the equivalent migration distances of reference bands R24, R50, and R79 in patterns of Marquis (outside slots) and Neepawa (center). Numbered patterns are for Canadian common spring wheat cultivars (left to right) Bishop, Concorde, Dundas, Glenlea, Huron, Kota, Laval 19, and Milton.

TABLE II
Relationships Used to Compute Relative Mobilities (Rm) by Multiple Reference Bands

Term	Definition	Eq. No.
Mobility relative to:		
reference band R24	$Q_i(x) = \dfrac{23.88\,(x_i)^a}{xR24^b}$	(1)
reference band R50	$R_i(x) = \dfrac{50.0\,(x_i)}{xR50^c}$	(2)
reference band R79	$S_i(x) = \dfrac{78.85\,(x_i)}{xR79^d}$	(3)
Weighting function for:		
gliadin bands between R24 and R50	$l_i(x) = \dfrac{R_i(x) - 23.88}{50.00 - 23.88}$	(4)
gliadin bands between R50 and R79	$h_i(x) = \dfrac{R_i(x) - 50.00}{78.95 - 50.00}$	(5)
Evaluate $[Rm = R_i(x)]$	Computed Relative Mobility	
if $\quad Rm < 23.88$	$Rm = Q_i(x)$	
$23.88 < Rm < 50.00$	$Rm = [1 - l_i(x)]\,Q_i(x) + l_i(x)\,R_i(x)$	(6)
$50.00 < Rm < 78.95$	$Rm = [1 - h_i(x)]\,R_i(x) + h_i(x)\,S_i(x)$	(7)
$Rm > 78.95$	$Rm = S_i(x)$	

[a] x_i = migration distance of ith sample gliadin band.
[b] $xR24$ = migration distance of band R24 in electrophoregram.
[c] $xR50$ = migration distance of band R50 in electrophoregram.
[d] $xR79$ = migration distance of band R79 in electrophoregram.

Figure 3 shows that standard deviation (SD) and coefficient of variability (CV) as a function of relative mobility for both normalization approaches is neither constant nor random, but varies systematically with the position of a band in the electrophoregram. Distinct SD and CV minima are reached in the vicinity of reference band positions, and Rm uncertainty increases with increasing distance from a reference band. This is most clearly indicated for the single reference band CV curve which varies in a symmetrical fashion about Rm = 50. Thus, while the absolute uncertainty (SD) in single reference band Rm for the fastest migrating gliadins is considerably higher than for low-mobility counterparts, the size of the relative error (CV) for bands at equal and opposite ends of the electrophoregram is similar.

The likely sources of error relate to localized variations in gel buffer composition, pH, voltage, and temperature that arise during the course of the electrophoresis period. In fact, the only time the gel is truly a homogeneous medium is before the run begins. Clearly then, the greater the number of standardizing marker bands applied across the gel in determining relative mobilities, the more the effects of these sources of error will be minimized.

As described above, low- and high-mobility reference bands R24 and R79 have a decreasing influence on the determination of relative mobilities for gliadin components with migration velocities approaching the intermediate reference band R50. This effect is well demonstrated in Figure 3 as the respective pairs of SD and CV curves all converge at Rm = 50 but become increasingly separated as distance from the center increases. In the immediate neighborhoods (± 4 Rm units) of the low- and high-mobility reference band positions, there is an average sevenfold difference in SD and CV values derived from the two standardization methods. The combined data for all Neepawa bands yield an average uncertainty in the determination of Rm by the single reference band
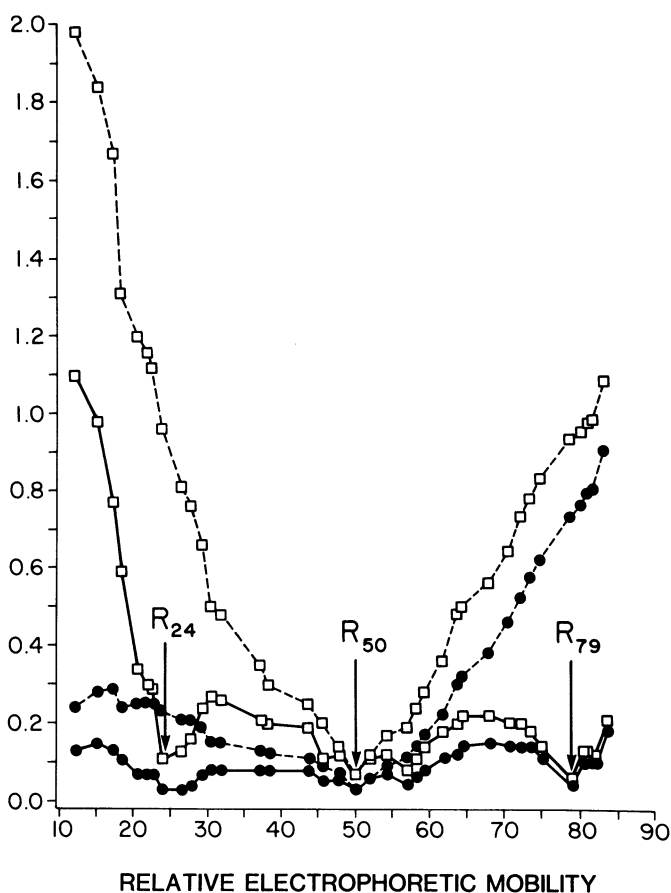
procedure (SD = 0.31, CV = 0.7%) more than three times greater than by multiple reference bands (SD = 0.09, CV = 0.2%). With the exception of bands possessing intermediate mobilities, the F-test confirmed that a highly significant difference exists in precision between the two sets of data. These results were invariably typical for the more than 500 single-kernel and bulk-grain samples that have been examined by PAGE to prepare a computerized catalog of gliadin electrophoregram data for cultivar identification (Sapirstein and Bushuk 1985a).

DISCUSSION

Significant differences notwithstanding, the results suggest that both single and multiple reference band normalization approaches are relatively effective to derive reproducible Rm values. A suitable question to ask is what level of precision is required for cultivar identification. This process typically involves matching an unknown or sample electrophoregram with all reference electrophoregrams encoded in the data base taken one at a time. For each paired comparison, assessments of band identity are inferred by comparing recorded Rm ± threshold. This threshold must be set wide enough to accept "truly" common protein components but not so wide that mismatches result between different protein components.

The problem is a classical one of minimizing both so-called type I (the rejection of true band identity) and type II (the acceptance of false gliadin band identity) errors when the null hypothesis is that no significant difference exists between the Rm of compared bands. Minimizing type I error can be achieved with relative ease by selecting a threshold value not less than the 95% confidence limit (95% CL), which for n determinations of mean Rm is given as:

$$95\% \text{ CL} = \pm t_{.05}\sqrt{SD^2/n},$$

where $t_{.05}$ is the critical value of Student's t on $(n-1)$ degrees of freedom. Unfortunately, the size of this threshold may conflict with the ability to detect the alternative hypothesis when it is true (i.e., minimizing type II error). While this problem can be avoided by excluding contributions from nonmatching bands in the cultivar identification strategy (Lookhart et al 1983), only differences between protein bands may be detected with certainty by electrophoresis.

For electrophoregrams run on the same gel slab, we have found that a separation of 0.5 Rm units is sufficient to visually distinguish different bands in adjacent patterns. For electrophoregrams so close together, it can be assumed that the difference between cultivars is completely genotypic, as experimental error is reduced to a minimum. However, to detect a significant difference based upon relative mobility data in a minimal replication experiment

Fig. 3. Standard deviation (●) and coefficient of variation (□) as a function of gliadin band relative electrophoretic mobility for cultivar Neepawa determined using one (- - -) and three (——) reference band standardization methods.

TABLE III
Standard Errors and Least Significant Differences for Relative Mobility Determination by One and Three Reference Band Standardization Methods[a]

Relative Mobility Range	Mean Number of Observations	One Reference Band		Three Reference Bands	
		SE	LSD	SE	LSD
10–20	188	0.11	0.70	0.05	0.31
20–30	380	0.11	0.67	0.04	0.27
30–40	290	0.09	0.54	0.06	0.35
40–50	373	0.06	0.34	0.05	0.31
50–60	423	0.07	0.41	0.06	0.34
60–70	334	0.15	0.90	0.07	0.45
70–80	397	0.32	1.97	0.06	0.36
80–90	175	0.45	2.75	0.09	0.57
10–90	2560	0.15	0.94	0.06	0.36

[a] Statistics are based on band relative mobility measurements for 77 sets of gliadin electrophoregrams for common and durum wheats in the cultivar identification data base of Sapirstein and Bushuk (1985a). Each set comprises three replications of electrophoregram data derived from different runs over a period of five months.

requires an uncertainty in the data substantially less than the difference to be detected. For example, the least significant difference (LSD) test can be used to compare mean Rm data for adjacent band positions. Where

$$\text{LSD} = t_{.05}\sqrt{2SD^2/n} \text{ or } t_{.05}\sqrt{2} \text{ (standard error)},$$

it can be determined using three replicates as a practical basis, that a standard error (SE) of <0.08 is required in order to detect a significant difference of 0.5 Rm units. This criterion is considerably less than typical values for Rm variability reported in the literature (Lookhart et al 1983, Zillman and Bushuk 1979) but well within the limits of error for most band mobilities calculated by multiple reference bands (Table III). By the single reference band approach, only for gliadins with intermediate mobilities (i.e., closest to reference band R50) is the variability sufficiently low to permit significant differences ($P = 0.05$) less than 0.5 Rm units to be detected between bands.

The data in Table III underscore as well the contrast in the level of uniformity in precision achieved by the two Rm measurement procedures. For the three reference band method, the LSD between bands varies 0.3 Rm units over the full Rm spectrum. By the single reference band approach, however, the LSD statistic deviates by more than 2 Rm units, depending on a band's position in the electrophoregram. This result indicates that a fixed-similarity threshold strategy to correlate protein band mobilities (Lookhart et al 1983) may be of questionable value in a computerized cultivar identification method based on the single reference band nomenclature. It is noteworthy as well, that differences between contiguous high mobility bands, given an average LSD value in the range of about 2 Rm units (Table III), are essentially undetectable by the standardization method based on one reference band.

It is clear that electrophoregram reproducibility is a critical factor when using relative mobilities to infer the identity of band positions on different gels. While an increase in sample size by replication will result in a narrowing of confidence limits and improved precision, this is generally not a practical solution when performing electrophoresis on a large number of samples. (Based upon average uncertainties in the determination of relative mobilities, the single reference band method requires about nine replications to obtain the equivalent level of precision achieved by the three reference band procedure using three replicates.) Accordingly, catalogs of gliadin electrophoregram data for cultivar identification incorporating few replicates are typical (e.g., $n = 1$, Autran and Bourdet 1975; $n = 2$, Jones et al 1982; $n = 1$, Zillman and Bushuk 1979).

Therefore, the implementation of a multiple reference band nomenclature for the determination of gliadin electrophoregram Rm is an effective strategy that will provide a satisfactory level of precision for computer-based comparative analysis of cultivar formulas, or a suitable basis for more automated methods to quantify PAGE results. In this regard, the feasibility of deriving reproducible Rm (and band intensity) data from computer-analyzed densitometric scanning profiles has been demonstrated (Sapirstein 1984). Exceptionally precise quantification will be required for a completely instrumental method of cultivar identification in wheat and other crops using electrophoretic methods; this requirement should be satisfied by normalizing densitometric data using multiple reference bands as described. This new Rm standardizing method should also facilitate better agreement for interlaboratory comparisons by fixing the scale of band relative mobilities at three separated points across the electrophoregram. Moreover, as reported in Sapirstein and Bushuk (1985b), when the uncertainty in band relative mobilities for a large population of genotypes is uniformly minimized, improved estimates of gliadin heterogeneity and an objective band classification can be realized from one dimensional PAGE data.

CONCLUSIONS

Many factors contribute to the variability in Rm measurements of bands in gliadin electrophoregrams despite the use of standardized methodology or the technical skill and experience of an operator. Thus, whereas the complex heterogeneity of gliadin electrophoregram composition is potentially well suited for wheat cultivar identification, rigorous standardization of protein band positional data is an important precondition for obtaining reliable results when comparing band relative mobilities from different electrophoregrams run on different gels. To satisfy this requirement, we have described a new gliadin-band nomenclature using three reference bands to determine relative electrophoretic mobility. The major advantage of this procedure is to improve the precision of results compared with the single-reference band approach by stabilizing the variability in Rm measurements at a low level across the entire electrophoregram field. The average uncertainty in the relative position of gliadin bands was reduced more than threefold with mean SD falling below 0.1 Rm units. The overall level of precision obtained by this method, implemented by a computer program, is substantially higher than that obtainable by single-reference band standardization.

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