Wheat Varietal Identification by Capillary Electrophoresis of Gliadins and High Molecular Weight Glutenin Subunits

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Cereal Chem. 71(5):397–402

ABSTRACT

The two main polyacrylamide gel electrophoresis (PAGE) methods used for wheat varietal fingerprinting have been low pH analysis (acid-PAGE) of the alcohol-soluble proteins, and sodium dodecyl sulfate (SDS-PAGE) of total gluten proteins denatured and extracted by the detergent SDS in the presence of reducing agents. Equivalent separations have now been achieved in the capillary electrophoresis format. The acid-PAGE separation was accomplished by free-solution capillary electrophoresis with a charge-reversed capillary. A separation analogous to SDS-PAGE for the analysis of reduced and denatured gluten was accomplished in the capillary format by including a soluble polyacrylamide sieving matrix in the buffer. This replaceable sieving matrix permitted analysis of multiple samples without cross-contamination between samples. The further addition of a small amount of organic solvent to the sieving matrix allowed for excellent resolution of the high molecular weight glutenin subunits that correlate with breadmaking quality. These techniques also possessed the inherent advantages of capillary electrophoresis: low mass requirements, fast separations, and quantitative analysis through on-capillary UV-detection.

Wheat is one of the most widely grown food grains. The grain endosperm is the source of white flour in the milling process, and the quality of products made from wheat flour-water doughs is often dependent upon the storage (gluten) proteins of the grain endosperm. For example, breadmaking requires a strong, elastic dough with suitable viscosity that will hold carbon dioxide in gas cells during fermentation; this characteristic is found in many bread wheat varieties (hexaploids with genome designation AABBDD). On the other hand, pasta doughs, predominantly produced from durum wheat varieties (tetraploids with genome AABB), must survive boiling without loss of cohesiveness.

The differences in quality associated with different varieties have long been known, and as the number of wheat varieties increased over the years, visual identification of varieties became unreliable. In recent years, quality differences in bread wheats have been correlated with particular groups or blocks of gluten proteins resulting from allelic genes. For example, high molecular weight glutenin subunits (HMW-GS) encoded by the *Glu-D1* loci are clearly correlated with quality through their allelic variants (Payne et al 1981, Payne 1987). Additionally, allelic variations at other loci have also shown correlations with quality (Metakovsky et al 1990). Because of the need for a more objective method for varietal identification, and a desire to exploit correlations of particular proteins in varieties with quality during breeding, the development of fast, accurate methods for wheat varietal identification has become highly important.

Various methods have been developed to address the issue of varietal identification and protein pattern recognition. Most of these were either chromatographic or electrophoretic techniques (Wrigley et al 1982; Bietz and Simpson 1992; Wrigley 1992; Autran, *in press;* Lafiandra et al, *in press*), and have included separations of the monomeric alcohol-soluble gliadins, sodium dodecyl sulfate (SDS)-soluble unreduced proteins (gliadins and polymeric glutenins), total proteins extracted with SDS after reduction of disulfide bonds (primarily gliadins plus glutenin subunits), and glutenin subunits (solubilized by reduction and extraction with SDS or other solvents after prior extraction of gliadins).

The most discriminating chromatographic technique has been reversed-phase high-performance liquid chromatography (RP-HPLC) (Bietz 1983, Bietz 1986, Courçoux et al 1992). Although chromatographic separations have been gaining in acceptance, electrophoretic techniques have remained the most popular procedures for analyzing wheat storage proteins. Electrophoretic separations are relatively simple, do not require expensive apparatus, and are familiar to most researchers. The main drawbacks are that gel preparation usually requires the handling of toxic acrylamide monomer, and quantitation is difficult because of the staining-destaining protocols necessary to visualize proteins after separation. One of the earliest high-resolution electrophoretic techniques separated gliadins (in the absence of detergents) at pH 3.1 in a starch or acrylamide gel with an aluminum lactate buffer system (Woychik et al 1961, Lee 1963, Autran and Bourdet 1975, Bushuk and Zillman 1978, Mecham et al 1985, Metakovsky and Novoselskaya 1991). The separation mechanism is based mainly upon the inherent charge of the gliadins, with some sieving contributed by the gel matrix. The advent of electrophoresis of reduced proteins in SDS polyacrylamide gels (SDS-PAGE) (Laemmli 1970) enabled high-resolution analyses of the normally disulfide-linked glutenin subunits (Bietz and Wall 1972). This method quickly became important for wheat protein analysis (Payne and Corfield 1979). Isoelectric focusing (IEF) has also been employed to separate wheat storage proteins (Morel and Autran 1990), especially as the first dimension for a two-dimensional electrophoretic analysis (Brown and Flavell 1981).

The development of capillary electrophoresis has introduced the capability for very fast electrophoretic separations (Jorgenson and Lukacs 1981). Narrow diameter capillaries are inherently anticonvective, thereby precluding the need to use gels to obtain high-resolution separations. On-line absorbance detection allows for direct quantitation, which as mentioned earlier, is difficult in gels where proteins must generally be stained after separation for quantitation. The separation of alcohol-soluble proteins has been demonstrated in the capillary format (Bietz and Simpson 1992, Bietz 1994). In this article, we describe two electrophoretic separations in the capillary format. One of these separations is analogous to the low pH gliadin separation and employs a cationic, polymer-coated capillary to minimize protein absorptive losses to the wall of the capillary (Wiktorowicz and Colburn 1990). The other separation is analogous to SDS-PAGE and employs an entangled polymer network rather than a rigid gel (Werner et al 1993). Both of these separations possessed resolution sufficient to allow differentiation of most wheat varieties. The

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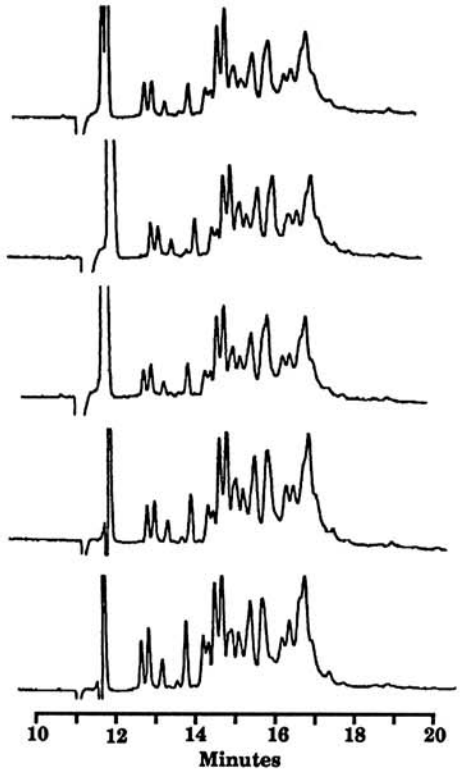

Fig. 1. Gliadin separations by capillary electrophoresis in aluminum lactate buffer. Patterns from five individual seeds of the cultivar Yecoro Rojo are shown. The capillary (72-cm total length, 50-cm separation length, and 50-μm i.d.) was coated with the Micro-Coat reagent and equilibrated with an aluminum-lactate buffer (pH 2.3). Samples were injected hydrodynamically and separated at −10,000 volts (7 μA). The large negative peaks (at 11–12 min) mark where uncharged molecules would migrate (electroendosmotic flow marker).

wheat varieties used in this study are all distinguishable by classical slab-gel techniques.

MATERIALS AND METHODS

Capillary Electrophoresis

Throughout this study, an Applied Biosystems 270A-HT capillary electrophoresis system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA) was employed. Gliadin analyses were performed using the charge-reversal reagent Micro-Coat (Applied Biosystems) in a capillary of 72-cm total length, 50-cm separation length, and 50-μm inner diameter. This reagent reverses the charge on the capillary surface, thereby actively repelling cationic proteins and diminishing protein adsorptive losses. When a capillary is coated with this reagent, the electrically driven bulk flow of buffer (electroendosmosis) opposes and exceeds protein migration. This results in the proteins being dragged past the detector by electroendomosis with the lower mobility (lower charge density) proteins eluting first. The buffer employed consisted of 50 mg of aluminum lactate (Fluka, Switzerland) and 650 μl of 90% lactic acid (Fluka) made up to a final volume of 30 ml with deionized water (pH 2.25). Separations of proteins denatured in SDS were performed using the ProSort SDS-Protein analysis kit (Applied Biosystems). This kit employs a replaceable, entangled polymer solution as the matrix for sieving SDS-denatured proteins in a capillary of 42-cm total length, 22-cm of separation length, and 55-μm inner diameter. The ProSort reagent was modified by the addition of 500 μl of 75% glycerol and 500 μl of methanol into 9 ml of reagent. (This must be prepared on a daily basis because of the evaporation of the methanol). Vial septa were placed on the buffer vials to prevent the evaporation of the methanol. The capillaries were thermostated to 30°C, and detection was at either 200 nm for the Micro-Coat experiments or 215 nm for the ProSort experiments.

Sample Preparation

Whole seeds were weighed and crushed with a hammer in glassine weighing paper. The crushed seeds were transferred to

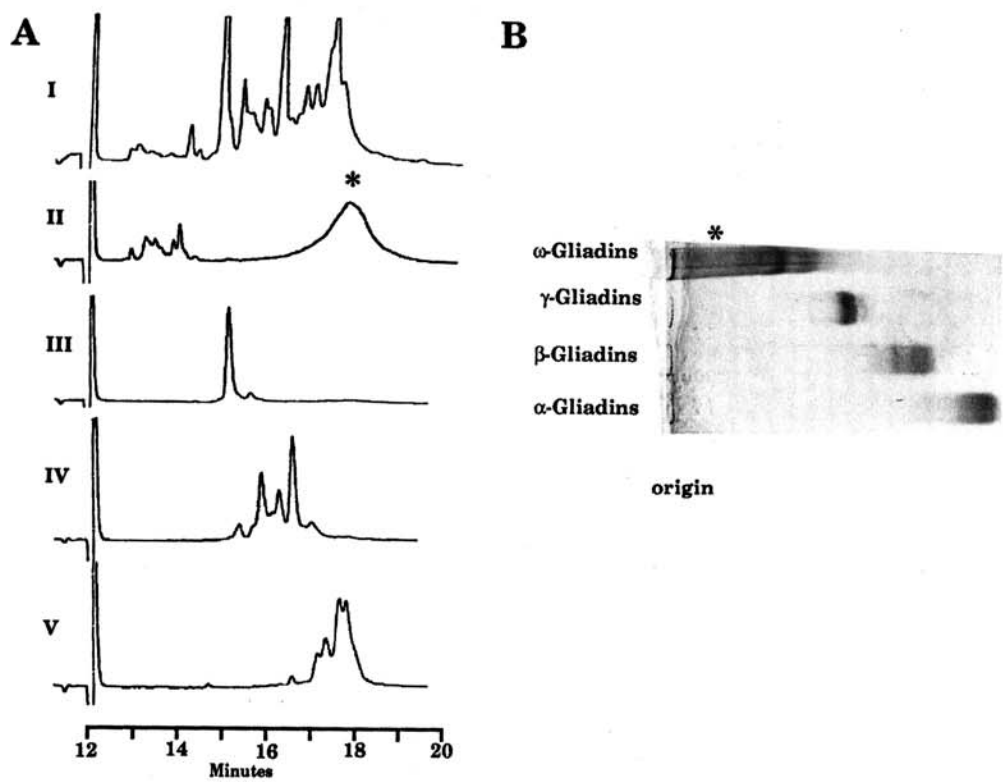

Fig. 2. Separation of purified gliadin components. (**A**) Electrophoregrams of (I) total gliadins extracted from Scout 66 seeds and purified gliadin fractions (II, ω-gliadins; III, γ-gliadins; IV β-gliadins; V, α-gliadins). (**B**) An aluminum-lactate gel pattern of the fractions. The acetic acid extracted glutenin (*) did not enter the lactate-PAGE, yet appeared to co-migrate with the α-gliadins by capillary electrophoresis.

1.5-ml snap-cap vials and extracted with either 70% ethanol (10 μl/mg) for gliadin analyses, or 1% SDS and 1% 2-mercaptoethanol (10 μl/mg) for total protein analyses. After a few hours of extraction, the supernatants were clarified by centrifugation and, in the case of the ethanol extractions, the supernatants were acidified by the addition of glacial acetic acid to a final volume of 1% before analysis. The SDS and 2-mercaptoethanol supernatants were placed in a boiling water bath for 5 to 10 min before analysis.

Purified gliadin fractions were prepared by gel-filtration chromatography. Proteins were extracted from flour with dilute acetic acid (0.1*M*). Gluten proteins were precipitated by adding concentrated sodium chloride solution to the extract until the salt concentration reached 0.15*M*. The salt-soluble albumins and globulins largely remained in solution. Fractionation of the precipitated proteins, after resolubilization in 0.01*M* acetic acid, was performed on Sephadex G-100 (Pharmacia) with 0.01*M* acetic acid as eluant. Column fractions were analyzed by aluminum-lactate gel electrophoresis before lyophilization and storage at −20°C. For capillary electrophoresis, these proteins were resuspended in 70% ethanol with 1% glacial acetic acid and analyzed on the charge-reversed capillary described previously. The gliadin fractions were also reanalyzed by standard aluminum-lactate gel electrophoresis (Lafiandra and Kasarda 1985) with a Protean II minigel apparatus (Bio-Rad, Hercules, CA).

RESULTS AND DISCUSSION

Micro-Coat Separations

Typical electrophoregrams for gliadins extracted from single whole seeds are shown in Figure 1. These electrophoregrams were derived from five seeds of the cultivar Yecoro Rojo prepared individually and analyzed by capillary electrophoresis using a charge-reversed capillary. These five samples illustrate the repro-

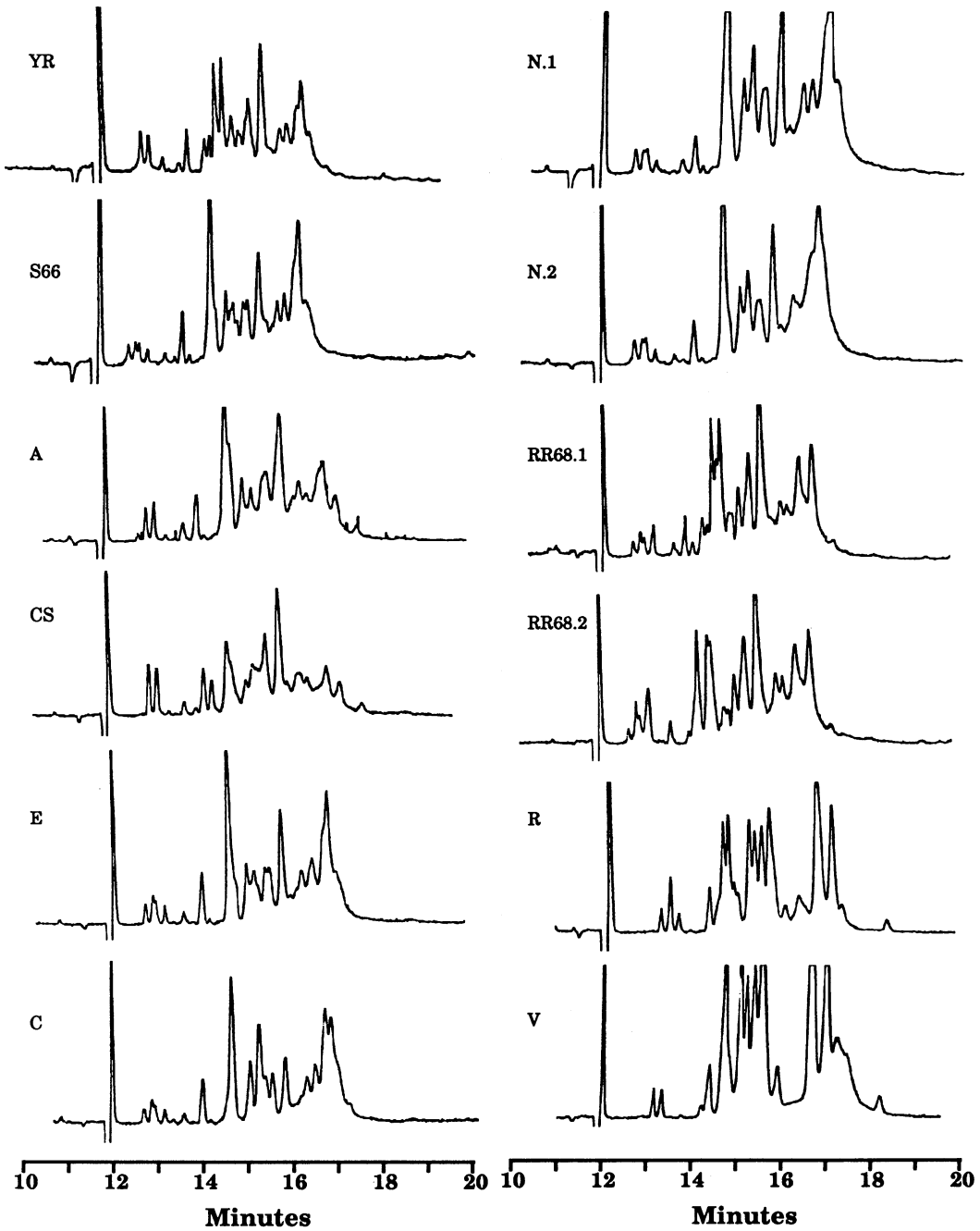

Fig. 3. Capillary electrophoresis of gliadins from single seeds of various wheat varieties (YR, Yecoro Rojo; S66, Scout 66; A, Anza; CS, Chinese Spring; E, Eagle; C, Cheyenne; N.1, Newton Biotype 1; N.2, Newton Biotype 2; RR68.1, Red River 68 Biotype 1; RR68.2, Red River 68 Biotype 2; R, Rugby; V, Vic). Electrophoretic conditions were identical to those described in Figure 1.

ducibility (relative standard deviation of <1% for migration times relative to a neutral marker) of the varietal pattern. To dissect the gliadin pattern further, and to compare it to traditional acid-PAGE (pH 3.1), purified gliadin fractions from the cultivar Scout 66 were analyzed by both capillary electrophoresis and acid-PAGE (Fig. 2). The gliadin fractions clearly have the same relative mobilities in both the capillary format (Fig. 2A) and the slab-gel format (Fig. 2B). For example, the high-mobility α-gliadins elute, as expected, at a later time in the capillary and migrate furthest in the slab-gel format. The one difference between these two techniques was seen for those glutenins extracted by 0.1*M* acetic acid (but minimally extracted by 70% ethanol) that coeluted to some extent with ω-gliadins upon gel filtration; that is, glutenin polymers elute largely in the void volume of BioGel P-100, but with some trailing into the resolved range, while ω-gliadins elute just beyond the void volume. These glutenin molecules are too large to freely enter a solid gel matrix, and they appear as a smear at the top of the gel. Conversely, in the capillary format, these glutenins appeared to migrate with the same mobility as the α-gliadins. This observation is in accord with earlier results from open tube moving-boundary electrophoresis (Jones et al 1959), where electrophoresis was performed in free solution and separations were observed with Schlieren optics. In addition, analysis of individually purified ω-gliadins demonstrated that the earlier eluting peaks (13–14.5 min, Fig. 2) were the ω-gliadins (data not shown).

Gliadins were extracted from 10 bread wheat cultivars and two durum wheat cultivars and analyzed by capillary electrophoresis to evaluate the potential of the method for varietal identification (Fig. 3). All 12 cultivars were easily distinguishable, demonstrating the potential of capillary electrophoresis for varietal fingerprinting.

ProSort Separations

When storage proteins are extracted with SDS in the presence of reducing agents, both gliadins and the normally disulfide-linked glutenin subunits are solubilized. SDS-PAGE has proven to be an important technique for analysis of storage proteins. An analogous separation is possible in the capillary format using a replaceable entangled-polymer network (Werner et al 1993) instead of a rigid, cross-linked gel. Figure 4 displays the electrophoregrams of five seeds extracted individually with SDS and 2-mercaptoethanol and analyzed using the ProSort SDS-protein analysis reagent. The electrophoregrams were reproducibile (relative standard deviations <3% for relative migration times over 8 hr), and the HMW-GS were very well resolved. The increase in the standard deviations, as compared to those found for the Micro-Coat separations (<1%), is due to the evaporation of the methanol added to the ProSort to resolve the HMW-GS. Identification of HMW-GS, using the Payne numbering system (Payne et al 1984), was achieved through comparison of various cultivars with known HMW-GS compositions.

One means to define the resolution of a separation is to analyze a complex mixture. This was done for the ProSort separation of HMW-GS by analyzing a mixture of Scout 66 (containing HMW-GS 2*, 2, 7, 9, 12) and Red River 68 (HMW-GS 1, 5, 7, 8, 10) extracts (Fig. 5). The *A*x-, *B*x-, and *D*x type subunits (1, 2*, 7, 5, and 2) and the *B*y subunits (8 and 9) were easily distinguishable in the resulting chromatogram, whereas the 1*D*y subunits (10 and 12) were recognizable, but not well resolved from other proteins (probably ω-gliadins). The resolution of these y-type HMW-GS, as well as the resolution of LMW-GS, which overlap with gliadins in the electrophoregram, might be improved by first extracting samples with SDS in the absence of reducing agents to remove gliadins before solubilizing the glutenin with SDS and reducing agents.

The potential for using the ProSort size-based separation for varietal fingerprinting and for identification of the complement of HMW-GS was explored by analyzing the SDS/2-mercaptoethanol extracts of the same 10 bread wheats and two durum wheats of Figure 3 (Fig. 6). All 12 cultivars displayed excellent resolution of the HMW-GS. In general, the relative elution order of the HMW-GS was the same in both the capillary and slab-gel formats, except for subunit 1, which eluted before subunit 5 in the capillary format. In addition, differences among the cultivars were noted for the elution patterns of the LMW-GS.

In this study, the effect of growth conditions on electrophoretic patterns was not investigated. The Yecoro Rojo sample chosen

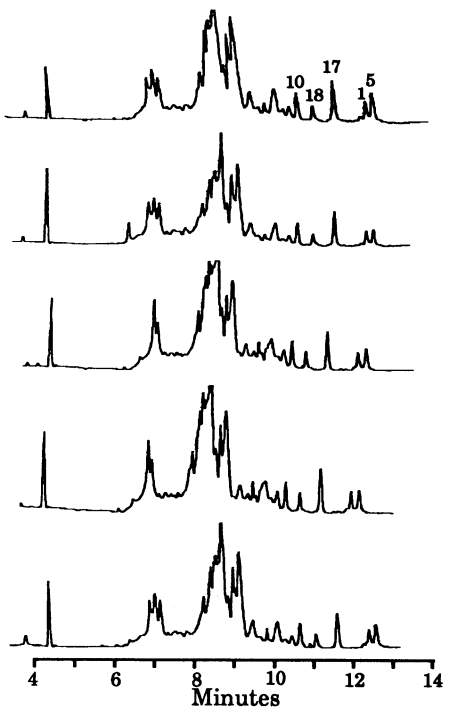

Fig. 4. Size-based fractionation of gluten proteins by capillary electrophoresis. The gluten from five seeds of the cultivar Yecoro Rojo was extracted as described previously. The ProSort matrix was modified by the addition of 75% glycerol and methanol (5% total volume of each). Samples were injected into the capillary (42-cm total length, 22-cm separation length, and 55-μm i.d.) electrokinetically and separated at −12,000 volts. In the top electrophoregram, the high molecular weight glutenin subunits are identified.

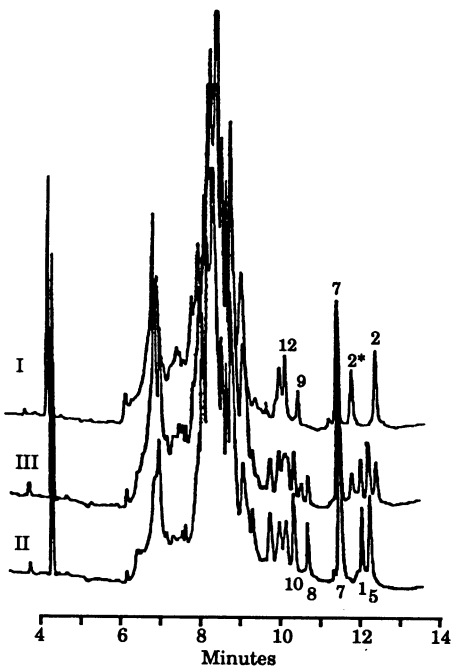

Fig. 5. Test of glutenin subunit resolution. Displayed are proteins from seeds of the cultivars (I) Scout 66 (subunits 2*, 2, 7, 9, 12) and (II) Red River 68 (subunits 1, 5, 7, 8, 10) as well as (III) a one-to-one mixture of the two cultivars. The electrophoretic conditions were identical to those of Figure 4.

for the reproducibility studies had been checked and was known to be free of biotypes. Initially, the cultivar Scout 66 was used to test reproducibility, but the results showed some variability in the patterns of individual seeds, apparently because of the presence of biotypes in the sample (data not shown).

CONCLUSION

Historically, slab-gel electrophoresis has been a very popular high-resolution technique for the analysis of wheat storage proteins. In this article, we have demonstrated that separations analogous to aluminum-lactate-PAGE and SDS-PAGE are possible in the capillary format. Both separations are rapid (analysis times of <30 min for each) and possessed resolution sufficient for varietal fingerprinting (Figs. 3 and 6). The resolution of HMW-GS using the modified ProSort matrix exceeded that typically seen for SDS-PAGE. Note subunit pairs 2/2* in Scout 66 and 17/18 in Yecoro Rojo (Fig. 6). In a mixture of two cultivars (Red River 68 and Scout 66) with a total of nine different HMW-GS, all of the subunits were resolved, although subunits 10 and 12 appeared to overlap slightly with the larger size ω-gliadins (Fig. 5), demonstrating further the capabilities of the ProSort matrix.

The capillary electrophoresis separations have the familiarity in component elution order and the high resolution of traditional slab-gel electrophoresis methods and are readily quantified through direct on-capillary detection. This improves the potential of the method for analyzing both qualitative and quantitative relationships with respect to quality and distinguishes this method from conventional slab-gel electrophoresis, in which protein staining is less accurate and less convenient for quantitation. At the same time, capillary electrophoresis is significantly faster than most of the high-performance liquid chromatographic methods developed for varietal identification.

ACKNOWLEDGMENTS

We thank Elva Adalsteins, WRRC, for preparing the gliadin fractions, and C. O. Qualset and Steve Jones, University of California-Davis, for increasing the Red River biotypes and providing some of the other samples.

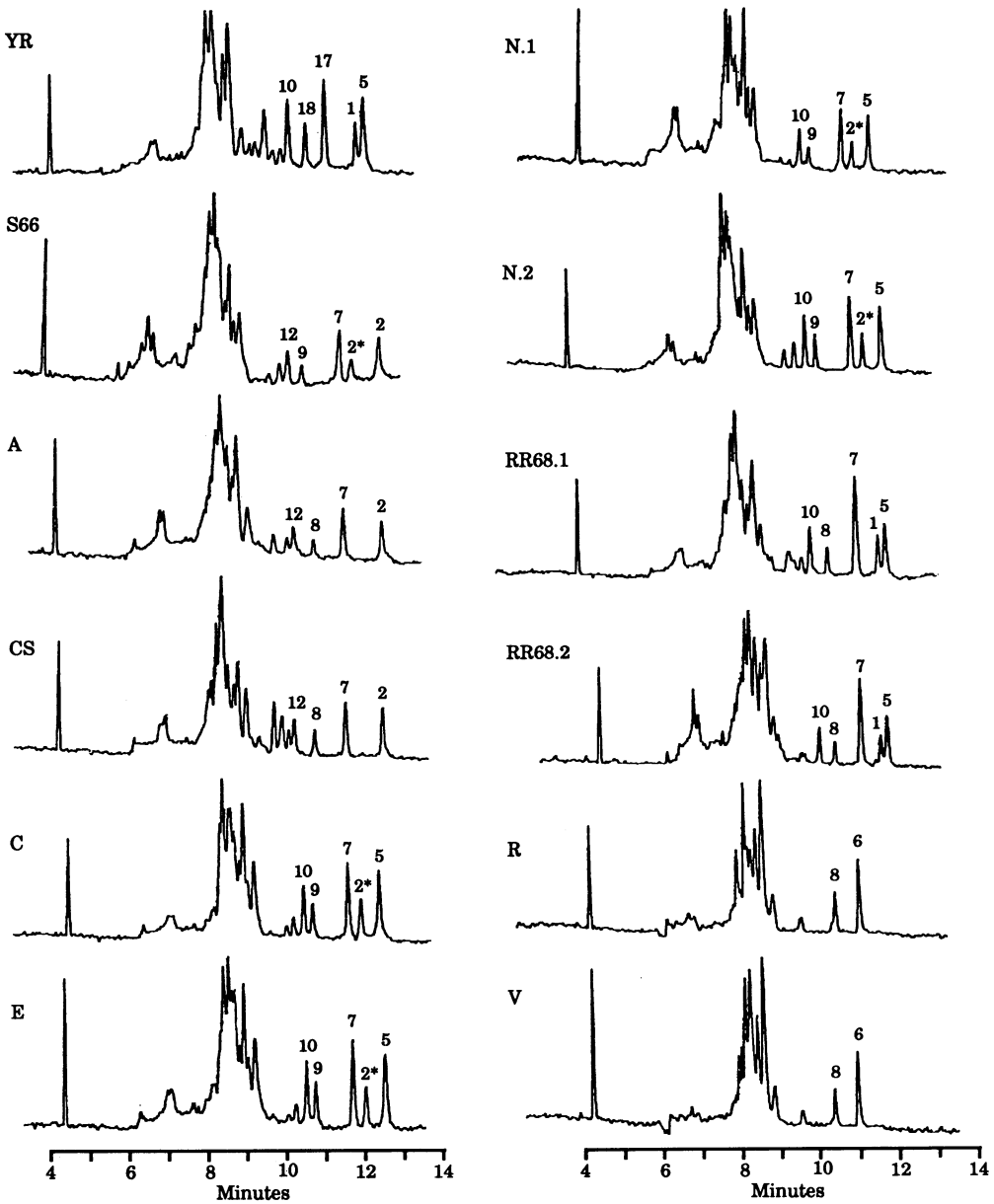

Fig. 6. Size-based capillary electrophoresis for distinguishing complements and varietal differences of high molecular weight glutenin subunits. Separation conditions were identical to those described in Figure 4. Electropherograms are displayed for the cultivars: YR, Yecoro Rojo; S66, Scout 66; A, Anza; CS, Chinese Spring; E, Eagle; C, Cheyenne; N.1, Newton Biotype 1; N.2, Newton Biotype 2; RR68.1, Red River 68 Biotype 1; RR68.2, Red River 68 Biotype 2; R, Rugby; and V, Vic. The high molecular weight glutenin subunits are identified by the appropriate numbers on the patterns.

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[Received December 27, 1993. Accepted May 6, 1994.]