Ferguson Plot Analysis of High Molecular Weight Glutenin Subunits by Capillary Electrophoresis

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ABSTRACT

The high molecular weight glutenin subunits of wheat endosperm migrate anomalously in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. This anomalous migration was studied by capillary electrophoresis employing an entangled polymer solution that contained SDS. The nonrigid solution nature of the sieving matrix allowed for the easy preparation of the different matrix concentrations required for the construction of a Ferguson plot. A Ferguson plot analysis of these proteins suggested that the high molecular weight glutenin subunits possessed frictional coefficients similar to those determined for standard proteins of the same size, indicating that anomalous migration in polyacrylamide gels was due to decreased binding of SDS and not to a unique structural conformation in the presence of SDS.

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The analysis of wheat storage proteins by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) has proven to be a valuable high-resolution technique that has demonstrated a special role for certain proteins in determining breadmaking quality (Lawrence and Shepherd 1980, Payne et al 1981, Ng and Bushuk 1989). This technique is ideal for characterizing the high molecular weight glutenin subunits (HMW-GS) of wheat gluten. SDS-PAGE has yielded molecular weights between 80,000 and 130,000 Da (Bietz and Wall 1972, Khan and Bushuk 1977, Bunce et al 1985, Graybosch and Morris 1990) for HMW-GS. Experiments employing an analytical ultracentrifuge have suggested that these estimates are too large (Hamauzu et al 1975). The recent cloning and sequencing of the genes encoding the HMW-GS has permitted the calculation of molecular weights based upon derived amino acid sequences that further supports the hypothesis that SDS-PAGE overestimates the molecular weight of HMW-GS (Sugiyama et al 1985; Thompson et al 1985; Halford et al 1987; Anderson et al 1989a,b; Halford et al 1992). It has been suggested that the anomalous migration behavior results from a highly stable secondary structure for HMW-GS that is resistant to denaturation in SDS and leads to a higher frictional drag in a gel matrix (Bunce et al 1985, Shani et al 1991).

SDS-PAGE relies upon two assumptions: 1) all proteins bind the same weight ratio of SDS (1.4 g of SDS per gram of protein) and thus have identical charge-to-mass ratios (Reynolds and Tanford 1970a); and 2) all SDS-protein complexes must assume the same conformation, the size of which varies linearly as a function of molecular weight (Reynolds and Tanford 1970b). Proteins that appear to migrate anomalously violate one or both of these assumptions. It is possible to get better molecular weight estimates for anomalous proteins by determining the relative migration of these proteins as a function of acrylamide gel concentration (Ferguson plot) (Ferguson 1964).

Ferguson (1964) observed that the logarithm of a protein's mobility varied linearly as a function of the gel concentration employed to determine the mobility. The slope of this relationship yielded a parameter called the retardation coefficient (Kr) that appeared to be proportional to the protein's molecular weight. The combination of these observations with Ogston's model (Ogston 1958) for sieving in a polymer network led to a stronger theoretical framework for gel electrophoresis (Rodbard and Chrambach 1970, Rodbard and Chrambach 1971, Tietz 1989). The modern-day Ferguson plot (logarithm of relative mobility versus gel concentration) that developed from these earlier observations yields retardation coefficients proportional to the square of the radii of the proteins. Standard curves may be constructed by plotting the logarithm of known protein molecular weights as a function of the square roots of the retardation coefficients. This Ferguson procedure has not been extensively employed because the preparation of the required multiple gel concentrations is relatively time-consuming. Studies have revealed that wheat storage proteins appear to have smaller molecular weights when analyzed by the Ferguson method as compared to standard SDS-PAGE (Hamauzu et al 1982, Hamauzu et al 1984).

The introduction of capillary electrophoresis has lead to rapid separations with on-line detection that precludes the need for postseparation staining to visualize proteins or DNA. Investigators have developed entangled-polymer solutions for separations of proteins and DNA in the capillary format (Zhu et al 1989, Bocek and Chrambach 1991, Sudor et al 1991, Ganzler et al 1992, Werner et al 1993a). These replaceable matrices have also been used to construct Ferguson plots for DNA and proteins (Bocek and Chrambach 1991, Sudor et al 1991, Werner et al 1993b). In this article, a commercially available capillary electrophoresis system has been employed to automatically generate the data required for Ferguson plot analysis of HMW-GS denatured in SDS. A replaceable polymer solution was employed, and the multiple gel concentrations were prepared by diluting the sieving matrix with buffer. It has been shown previously that this matrix allowed high-resolution analyses of HMW-GS in the capillary format (Werner et al 1994).

THEORETICAL CONSIDERATIONS

The relationship between protein mobility (μ) and sieving matrix concentration (T) may be written as (Tietz 1989):

$$\text{Log}(\mu) = \text{Log}(\mu_o) - Kr(T) \quad (1)$$

where μ_o is the free solution mobility (zero concentration of sieving matrix) and Kr is the retardation coefficient. The relationship between Kr and a protein's radius has been derived from Ogston's model as follows (Tietz 1989):

$$Kr = \pi l'(r + R)^2 \times 10^{-16} \quad (2)$$

where l' is the matrix fiber length (cm/gm), r is the fiber radius (nm), and R is the protein's radius (nm). Equation 2 describes the relationship between the retardation coefficient and the size of the proteins. Historically, a standard curve may be constructed by plotting the logarithm of protein molecular weight as a function of the square root of the retardation coefficient. This relationship explains why the Ferguson method is useful for analyzing proteins irrespective of their charge-to-mass ratios.

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MATERIALS AND METHODS

Materials

All capillary electrophoresis experiments were performed on a Perkin Elmer ABI model 270 capillary electrophoresis system employing the ProSort SDS-protein analysis kit as supplied by the manufacturer (Perkin Elmer Corp., Applied Biosystems Division, Foster City, CA). The ProSort sieving matrix is a formulation based upon linear polyacrylamide containing SDS. The kit also contained diluent buffer for making up different concentrations of the sieving matrix, capillaries, as well as a mixture of seven proteins that were used as standards.

Experimental Design

To generate a Ferguson plot, the proteins of interest as well as standards must be analyzed at several different concentrations of the sieving matrix (50, 67, and 80%). This was accomplished by diluting the ProSort analysis reagent with a buffer solution supplied in the kit. This study differed from a previous one (Werner et al 1994) in that the ProSort reagent was not modified by the addition of glycerol and methanol. The underivatized fused-silica capillary (42 cm total length, 22 cm separation length, and 55 mm i.d.) (Perkin Elmer, ABD) was first flushed with 0.1*N* sodium hydroxide, followed with deionized water, and finally equilibrated with the lowest concentration (50%) of the ProSort SDS-protein analysis reagent. Samples were injected electrokinetically (4 sec at −5 kV) onto the capillary and separated with an applied voltage of −12 kV (285 V/cm) at 30°C. The detector was set at 215 nm. Standards and samples were analyzed as a set at each matrix concentration before the instrument automatically reequilibrated the capillary with the next matrix concentration and reanalyzed the standards and samples. Relative migrations were calculated by dividing the migration time of a reference marker (mellitic acid) by the migration times of the respective proteins. The reproducibility for relative migration times using entangled polymer networks is <1.0% relative standard deviation (RSD) (Werner et al 1993a).

Sample Preparation

Whole seeds of the cultivars Scout 66 and Red River 68 were weighed and crushed using a hammer and glassine weighing paper. The crushed seeds were transferred to 1.5-ml snap-cap vials and extracted with 1% SDS and 1% 2-mercaptoethanol (10 μl/mg). After ~5 hr of extraction, the supernatents were clarified by centrifugation and were placed in a boiling water bath for 5–10 min before analysis. The standards were prepared by the addition of 300 μl of 2% SDS and 2% 2-mercaptoethanol (1 mg/ml of final protein concentration) and placed in a boiling water bath for 5–10 min.

RESULTS

Figure 1 contains electropherograms of the total storage proteins from the cultivar Scout 66 analyzed at four different concentrations of the ProSort sieving matrix. The identification of the specific HMW-GS has been discussed previously (Werner et al 1994) and involved the comparison of electropherograms from numerous cultivars of known HMW-GS composition. Relative migration times for each HMW-GS were calculated by dividing the migration time of the marker by the migration time of the protein. Figure 2 shows a Ferguson plot comparing the HMW-GS from Scout 66 to standard proteins. The relative migration times used for the construction of the Ferguson plot represents the average relative migration times derived from two injections of each sample at each matrix concentration. A similar plot was made for the HMW-GS from the cultivar Red River 68. In Figure 2, the data for the HMW-GS intersected the *y*-axis at values lower than those of the standards. The retardation coefficients were calculated from the slopes of the data.

As discussed earlier (Eq. 2), the retardation coefficients are proportional to the square of the protein's radii. A standard curve was constructed by plotting the square roots of the retardation coefficients against the logarithm of the molecular weights for standard proteins (Fig. 3). On this graph, the square root of the retardation coefficients for the HMW-GS of the cultivar Scout 66 were superimposed. The HMW-GS have molecular weights between bovine serum albumin (66,000 Da) and phosphorylase B (97,400 Da). This range is much smaller than that typically seen by SDS-PAGE (80,000–130,000 Da). The HMW-GS of both Scout 66 and Red River 68 were analyzed, and the average molecular weights (derived from three separate Ferguson plots) and the relative standard deviations are listed with the molecular weights based upon derived amino acid sequences (Table I).

DISCUSSION

Capillary electrophoresis in conjunction with entangled polymer solutions possesses many advantages for analyzing proteins by the Ferguson plot method. Multiple gel concentrations are made by simply diluting a stock solution, which may be changed automatically using a commercial capillary electrophoresis system. The use of a stock solution of sieving matrix that is simply diluted ensures that the dimensions of the sieving fibers (length and diameter) remain constant, which makes the determination of the retardation coefficients strictly a function of the protein's radii. This is only assumed in the slab gel format where polymerization conditions are slightly altered (varying concentrations of acrylamide monomer) to generate gels of different concentrations. The use of an on-capillary detector with a data system precludes the

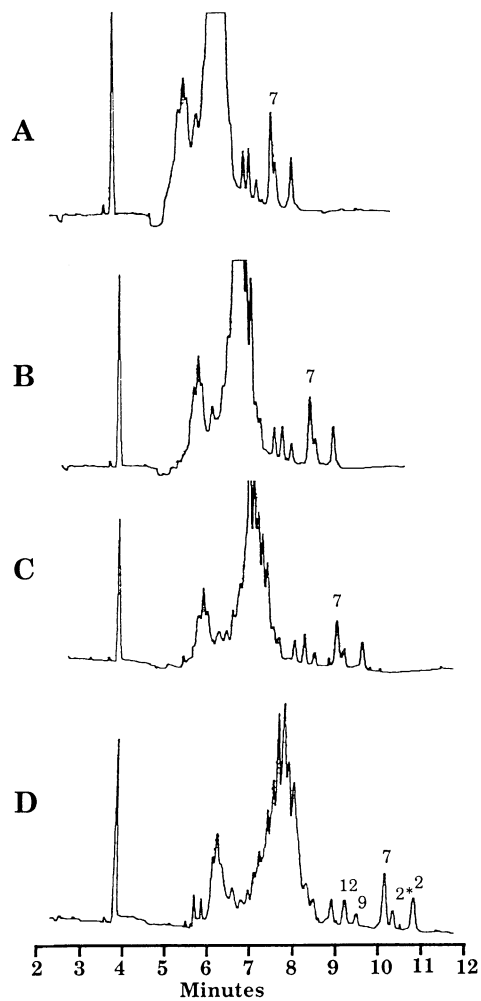

Fig. 1. Electropherograms for Scout 66 gluten. Total storage proteins from the cultivar Scout 66 were analyzed at four different dilutions of the ProSort sieving matrix: **(A)** 50%, **(B)** 67%, **(C)** 80%, and **(D)** 100%. High molecular weight glutenin subunits are identified in the last electropherogram (100% ProSort). Subunit 1BX7 is labeled 7 in all electropherograms as a reference point.

need for separate staining, destaining, and gel-measuring steps, thereby speeding up a time-consuming procedure.

In an attempt to understand the anomalous migration of HMW-GS in SDS-PAGE, the Ferguson plot method was employed to analyze these proteins. The analysis provides a parameter called the retardation coefficient *(Kr),* which is a function of the size and shape (frictional coefficient) of the protein, but not its charge. On a Ferguson plot (logarithm of relative migration versus the sieving matrix concentration), it was interesting to note that the standard proteins intersected the *y*-axis over a small range there by supporting the assumption of SDS-PAGE, namely that proteins bind the same weight ratio of SDS. The data for HMW-GS, on the other hand, intersected the *y*-axis at a point lower than well-characterized proteins typically used as standards. This indicated that the SDS-denatured HMW-GS possessed lower charge-to-frictional coefficient ratios than did standard proteins denatured in SDS. Preliminary experiments supported this conclusion when it was found that the SDS denatured HMW-GS possessed lower mobilities in free solution (absence of any sieving matrix) than did standard SDS denatured proteins (data not shown). It should also be noted, that the Ferguson plots for the HMW-GS did not intersect the *y*-axis at a single point, which was contrary to the results observed in a slab-based analysis (Hamauzu et al 1982). At this time, it is difficult to explain this discrepancy. However, it may be partly due to the fact that in the slab gel experiments, different polymerization conditions had to be used in constructing the different gels, which may have resulted in slightly different gel-fiber dimensions, which could influence the values obtained for the retardation coefficients and the *y*-intercepts. This phenomenon would not have been observed in the CE experiments where it was known that the fiber dimensions did not change.

Analysis of the retardation coefficients determined from Ferguson plots (Figs. 2 and 3, Table I) yielded molecular weight estimates for the HMW-GS very similar to those predicted from derived amino acid sequences. This suggests that the HMW-GS denatured in SDS have frictional coefficients very similar to standard proteins of the same molecular weights. The data displayed in Table I suggest that the Ferguson plot technique employed in this study does not have the precision expected in an analytical technique (%RSD <1.0). It is interesting to note that subunit 7 in Scout 66 yielded a molecular weight of 84,000 Da and that of Red

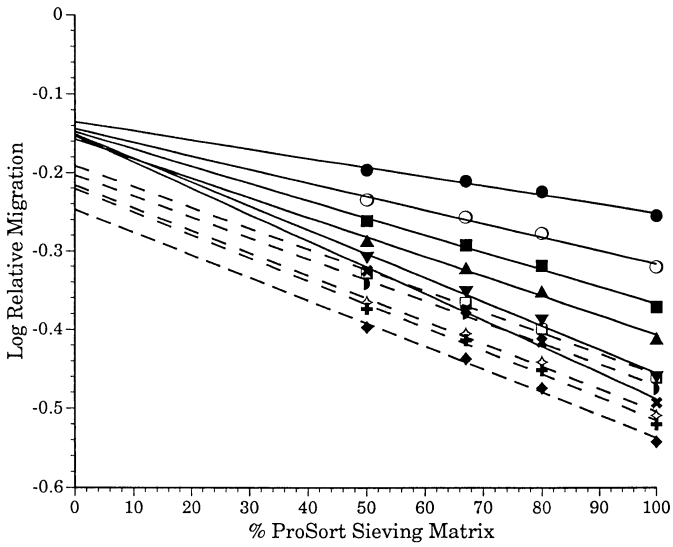

Fig. 2. Ferguson plots for standard proteins and high molecular weight glutenin subunits derived from Scout 66. Logarithm of relative migrations for the standard proteins (α-lactalbumin (●), carbonic anhydrase (○), ovalbumin (■), bovine serum albumin (▲), phosphorylase b (▼), and β-galactosidase (×); high molecular weight subunits from Scout 66: 2* (+), 2 (◆), 7 (◇), 9 (◗), and 12 (□) determined at four different concentrations of the ProSort matrix and plotted against the relative concentration of the ProSort matrix.

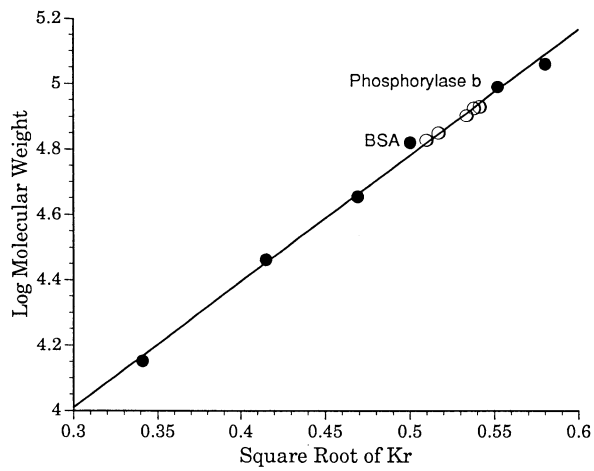

Fig. 3. Standard curve for molecular weight determinations. The square roots of the retardation coefficients determined for the (●) standard proteins: α-lactalbumin (14,000 Da), carbonic anhydrase (29,000 Da), ovalbumin (45,000 Da), bovine serum albumin (66,000 Da), phosphorylase B 97,400 Da), and β-galactosidase (116,000 Da) and for the high molecular weight subunits from Scout 66 (○) and plotted against the logarithm of molecular weights for standard proteins. Data were fit with a linear regression. Square root of the retardation coefficients of the high molecular weight glutenin subunits are superimposed on this line. Standard proteins bovine serum albumin (BSA) and phosphoprylase B are identified for reference.

TABLE I
Analysis of the High Molecular Weight Glutenin Subunits Derived from Three Separate Ferguson Plots

Subunits[a]	Molecular Weight (Derived from DNA Sequences)[b] (Daltons)	Molecular Weight SDS-PAGE[c] (Daltons)	Molecular Weight (Capillary Electrophoresis Ferguson) (Daltons)	Relative Standard Deviations, % ($n = 3$)
2*	86,300	136,000	89,000	5.1
2	87,200	134,000	89,000	11
7	83,100	115,000	84,000	6
9	73,000	95,700	71,000	4.3
12	68,700	91,200	71,000	4.5
1	87,700	149,000	89,000	6.6
5	87,200	128,000	86,000	12
7	83,100	115,000	88,000	8
8	...	101,000	76,000	9.7
10	67,500	92,100	71,000	7.8

[a]Subunits 2*, 2, 7, 9, 12 were from Scout 66 and subunits 1, 5, 7, 8, 10 were from Red River 68.
[b]Sources for the DNA sequences were referenced in text.
[c]Ng and Bushuk (1989).

River 68 had a molecular weight of 88,000 Da. These values are not statistically significant from one another, nor are they different from the molecular weight derived from DNA sequences (83,100 Da). It was also demonstrated in an earlier study (Werner et al 1994) that when the total gluten from these two cultivars was mixed and injected into a capillary that the subunit 7 from both varieties comigrated, suggesting that they were in fact the same protein. The use of Ferguson plots yields better molecular weight estimates for the HMW-GS than does SDS-PAGE at a single gel concentration. However, the technique is not precise enough to allow differentiation or identification of the HMW-GS, nor the detection of the presence or absence of small amounts of posttranslational modifications (carbohydrates), nor protein degradation (cleavage of N- or C-terminal amino acids).

From all of the data, it may be concluded that the anomalous migration of the HMW-GS in gels is due to a decreased binding of SDS to these proteins, and not to any unusual conformations that result in larger than expected frictional coefficients. This proposed decreased binding of SDS by the HMW-GS is in contrast to earlier equilibrium dialysis experiments which showed identical SDS binding for glutenin and standard proteins (Bietz and Wall 1972). In that study, the investigators obtained saturation levels of SDS significantly lower than what had been reported earlier (0.9 vs. 1.4 g of SDS/g of protein). It may be questioned whether or not SDS saturation was achieved in these experiments. If not, then potential differential binding of SDS by glutenin may not have been observed. In addition, the investigators employed total glutenin, which may not have yielded an accurate value for just the HMW-GS. When the *y*-intercepts from the Ferguson plots (Fig. 1) are converted to electrophoretic mobilities, it appears that the HMW-GS possess mobilities about 15–20% less than the standard proteins. Because mobilities are dependent upon the ratios of charge-to-frictional coefficient, and it has been demonstrated that the HMW-GS have frictional coefficients similar to standard proteins of the same size, then it may be concluded that the HMW-GS possess 15–20% less charge (bound SDS) than do standard proteins denatured in SDS. The decreased binding efficiency for SDS may be due to the unusual amino acid sequences of these proteins. The possibility of an unusual conformation for the HMW-GS in the absence of SDS is not, however, ruled out by these experiments.

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