Identification of United States Barley Cultivars by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Hordeins[1]

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ABSTRACT

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The possibility of identifying United States barley cultivars by the sodium dodecyl sulfate electrophoretic patterns of the hordein proteins was studied. Hordein polypeptides were extracted with 55% (v/v) isopropanol and 2% (v/v) 2-mercaptoethanol. Electrophoresis of samples prepared by direct dilution of the extract solution with sample buffer yielded more consistent electrophoregrams than those generated from precipitated hordeins. This was true whether the precipitated, native hordeins were dissolved directly in sample buffer or if they were alkylated before solubilization. Use of a sensitive silver stain allowed us to use lower amounts of protein, which may have made alkylation unnecessary for good band resolution. Analysis times and operating costs were minimized by use of a mini-electrophoretic vertical slab gel apparatus. Fifty-five barley cultivars commonly grown in the United States were analyzed, and they yielded 34 different hordein patterns. Twenty-four cultivars gave unique patterns, whereas each of the others fell into one of 10 groups, each of which comprised from two to seven cultivars.

Key words: Biotypes, Cultivar identification

The correct identification of barley cultivars is important to different groups, especially to the malting and brewing industries, owing to the differential suitabilities of the cultivars for processing. Examination of grain morphological characteristics is the standard method of identifying barley cultivars, but not all cultivars can be distinguished on this basis. Several biochemical techniques (Cooke 1984, Wrigley et al 1982) have been used to augment morphological examination, and most of them rely on variations among the prolamins (hordeins), the alcohol-soluble seed storage proteins.

Hordeins have been divided into four groups, A, B, C, and D, on the basis of their molecular weights and on differences in their amino acid compositions (Shewry and Miflin 1985). The B (mol wt 32,400–45,000) and C (mol wt 49,000–72,000) groups, the major hordeins, are of intermediate sizes, and are encoded by the multigenic loci *Hor*2 and *Hor*1, respectively, which are located on chromosome 5. The D hordeins are minor components with higher molecular weights (mol wt ~ 105,000) and are encoded by the *Hor*3 locus (Kreis et al 1984). The A hordeins are lower molecular weight (mol wt <20,000) proteins and may not be true storage proteins (Miflin and Shewry 1977).

Hordeins have been separated on the bases of their electrophoretic mobilities using starch gel electrophoresis (Favret et al 1970, Autran and Scriban 1977, Shewry et al 1978a), polyacrylamide gel electrophoresis (PAGE) at acid pH (Shewry et al 1978a; Marchylo and Laberge 1980, 1981; Cooke and Cliff 1983; Gebre et al 1986), and sodium dodecyl sulfate (SDS) PAGE (Shewry et al 1978a,b; Kapala 1981; Curtis and Chadwick 1983; Montembault et al 1983; Smith and Payne 1984). Separations have also been achieved on the basis of differences in the isoelectric points of the hordeins by isoelectric focusing (Favret et al 1970, Shewry et al 1978a) and on their different hydrophobicities by reversed-phase high-performance liquid chromotography (Marchylo and Kruger 1984, 1985).

These methods allowed the identification of many European, Australian, and Canadian barley cultivars, but less attention has been given to commercially important barleys grown in the United States.

This paper reports our examination, by SDS-PAGE, of the hordein patterns of 55 barley cultivars commonly grown in the United States. Our use of a sensitive silver-staining technique allowed us to load lesser amounts of protein onto the gels and resulted in increased resolution of hordein bands.

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MATERIALS AND METHODS

Barley grain samples were supplied by Michael P. Davis (American Malting Barley Association, Milwaukee, WI), who obtained them from barley breeders located throughout the important barley-producing areas of the United States. Multiple samples of each cultivar, grown in at least two different environments, were analyzed.

Grain samples (20 g) were ground in a Udy Cyclone sample mill (Udy Corp., Fort Collins, CO) to pass through a 1-mm sieve. Alternatively, single seeds were crushed with pliers and pulverized to a fine powder with a mortar and pestle.

Hordein Isolation

Extraction. A pulverized single seed or a 40-mg flour sample was extracted with 1 ml of 55% (v/v) aqueous isopropanol containing 2% (v/v) 2-mercaptoethanol in a 10 × 75 mm Pyrex tube or a 1.5-ml polypropylene microfuge tube. Extraction was at 60°C for 30 min in a sonication bath with vortex mixing every 10 min. The extracts were then centrifuged at 20°C at 5,000 × g for 10 min (Pyrex tubes) or 8,400 × g for 4 min (microfuge tubes). The supernatants were decanted and saved.

Preparation of Hordeins for Electrophoresis

Salt precipitation. Hordeins were precipitated from extract supernatants by the addition of an equal volume of ice-cold 1.0 *M* NaCl. After 20 min in an ice bath, the precipitated hordein was recovered by centrifugation (5,000 × g, 10 min, 4°C) and rinsed with ice-cold deionized, distilled water (ddH_2O).

Dialysis precipitation. Supernatants were exhaustively dialyzed against at least a one-million-fold volume of ddH_2O at 4°C in Spectrapor 8,000 molecular weight cut-off dialyzer tubing (Sprectrum Medical Industries, Inc., Los Angeles, CA). The precipitated hordeins were collected by centrifugation (5,000 × g, 10 min, 4°C).

Alkylation. Precipitated hordeins were alkylated (carboxyamidomethylated) as described by Brinegar and Peterson (1982). Alkylated protein was precipitated from solution (0.87 ml) by the addition of 80 μl of 6 *N* HCl and 9 ml of ice-cold 0.55 *M* NaCl. After 20 min on ice, the hordeins were collected by centrifugation (10,000 × g, 10 min, 4°C). Alternatively, alkylated proteins were precipitated by exhaustive dialysis against ddH_2O at 4°C and recovered by centrifugation (5,000 × g, 10 min, 4°C).

Precipitated hordein samples, native or alkylated, were dissolved in 4.3 ml of SDS sample buffer by heating for 5–10 min in a boiling H_2O bath. SDS sample buffer contained 2% (w/v) SDS (electrophoresis grade, Bethesda Research Laboratories, Gaithersburg, MD), 10% (w/v) sucrose, 0.02% (w/v) bromophenol blue, 0.5 m*M* disodium ethylenediaminetetraacetic acid (Na_2EDTA), 50 m*M* dithiothreitol, and 0.025 *M* Tris-HCl, pH 6.8. Samples were stored frozen at −20 or −80°C until needed.

Rapid preparation method. Extraction supernatants were diluted eight-fold with SDS sample buffer and heated in a boiling water bath for 10 min.

Electrophoresis

SDS-PAGE was performed using a modification of the discontinuous gel method of Laemmli (1970). The stacking gel layers contained 4% (w/v) acrylamide (Electran grade 1, BDH Chemical Ltd., Poole, England), 0.11% (w/v) *N,N'*-methylene-bisacrylamide (BIS) (Bio-Rad Laboratories, Richmond, CA), 0.1% (w/v) SDS, 0.5 m*M* Na_2EDTA, and 0.125 *M* Tris-HCl, pH 6.8. The separating gels contained 0.1% (w/v) SDS, 0.5 m*M* Na_2EDTA, 0.375 *M* Tris-HCl, pH 8.8, and either 13% (w/v) acrylamide/0.24% (w/v) BIS or 15% (w/v) acrylamide/0.28% (w/v) BIS. The running buffer contained 0.19 *M* glycine, 0.1% (w/v) SDS, 1.0 m*M* Na_2EDTA, and 25 m*M* Tris-HCl pH 8.3. All gels were polymerized using 0.075% (w/v) ammonium persulfate and 0.038% (v/v) *N,N,N',N'*-tetramethylethylenediamine (Bio-Rad).

Nineteen samples (2.5 μl each or 5 μl each for rapid preparation extracts) were loaded onto each gel slab and were subjected to electrophoresis at 20 mA constant current per 14 × 12 × 0.075 cm slab on a Bio-Rad model 22 dual slab electrophoresis cell. The gels were run until 1 hr after the bromophenol blue dye had reached the bottom of the gel. Alternatively, 14 samples (0.8 μl each or 1.6 μl each for rapid preparation extracts) were run for about 1 hr on a mini-slab (8.0 × 6.0 × 0.075 cm) gel at 20 mA constant current, using a Bio-Rad model 360 mini vertical slab cell. Low-molecular-weight standard proteins from Sigma Chemical Co. (St. Louis, MO) were prepared according to the manufacturer's instructions and run on one lane of each gel.

Staining and Storage of Gels

Silver stain. Following electrophoresis, gels were immediately fixed in a 50:10:40 (v/v) solution of methanol, acetic acid, and water or in 12% (w/v) trichloroacetic acid (TCA) for a minimum of 1 hr, briefly rinsed in H_2O, and cross-linked with 8% (v/v) glutaraldehyde for 1 hr. The gels were then washed with three changes of ddH_2O and stained with silver nitrate by the method of Oakley et al (1980) as modified by Eschenbruch and Burk (1982). Color development was stopped when the desired band intensity was reached by washing the gel in either ddH_2O or 10% (v/v) acetic acid. The total time for silver staining a gel was approximately 3 hr, although several gels could be stained at the same time provided each gel was incubated in its own silver nitrate solution. Gels could be left overnight in either of the initial fixative solutions or in ddH_2O after cross-linking with glutaraldehyde.

Coomassie blue stain. Gels were fixed in a 50:10:40 (v/v) solution of methanol, acetic acid, and water for 1 hr, rinsed 30 min in ddH_2O, and stained overnight in 15% (w/v) TCA containing 0.0027% (w/v) Coomassie Brilliant Blue R-250 (CBBR). The staining solution was prepared by diluting 0.4 ml of 1.0% (w/v) CBBR in ethanol to 150 ml with 15% (w/v) TCA in H_2O. Destaining was not necessary. Alternatively, unfixed gels were stained overnight in 12% (w/v) TCA containing 0.04% (w/v) CBBR and destained at 4°C in 12% (w/v) TCA.

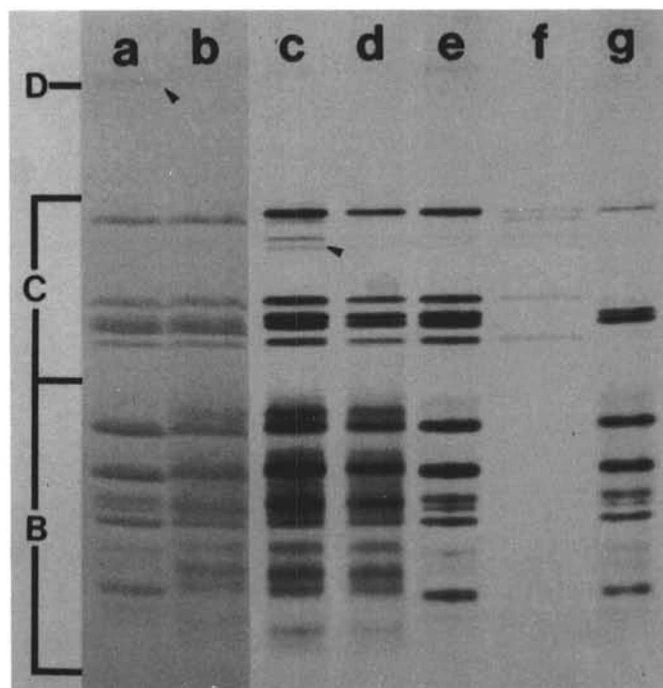

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of Morex flour samples showing the effects of extraction and staining methods. **a,** Salt-precipitated, alkylated, stained with Coomassie Brilliant Blue R-250. **b,** Salt-precipitated, nonalkylated, stained with Coomassie Brilliant Blue R-250. **c,** Rapid preparation, silver stained; arrows indicate bands that were consistently seen only with this method. **d,** Salt-precipitated, nonalkylated, silver stained. **e,** Salt-precipitated, alkylated, silver stained. **f,** Supernatant of dialysis-precipitated hordein, silver stained. **g,** Dialysis-precipitated, alkylated, silver stained. Samples were run from the cathode (top) to the anode.

Gel storage. After staining, gels were routinely air-dried at room temperature between two sheets of wetted, untreated cellophane, type Put-76, #128 (Olin Corp., Pisgah Forest, NC), clamped in an acrylic plastic frame.

RESULTS AND DISCUSSION

Hordein Isolations

Shewry et al (1978b) showed that hordeins were extracted as

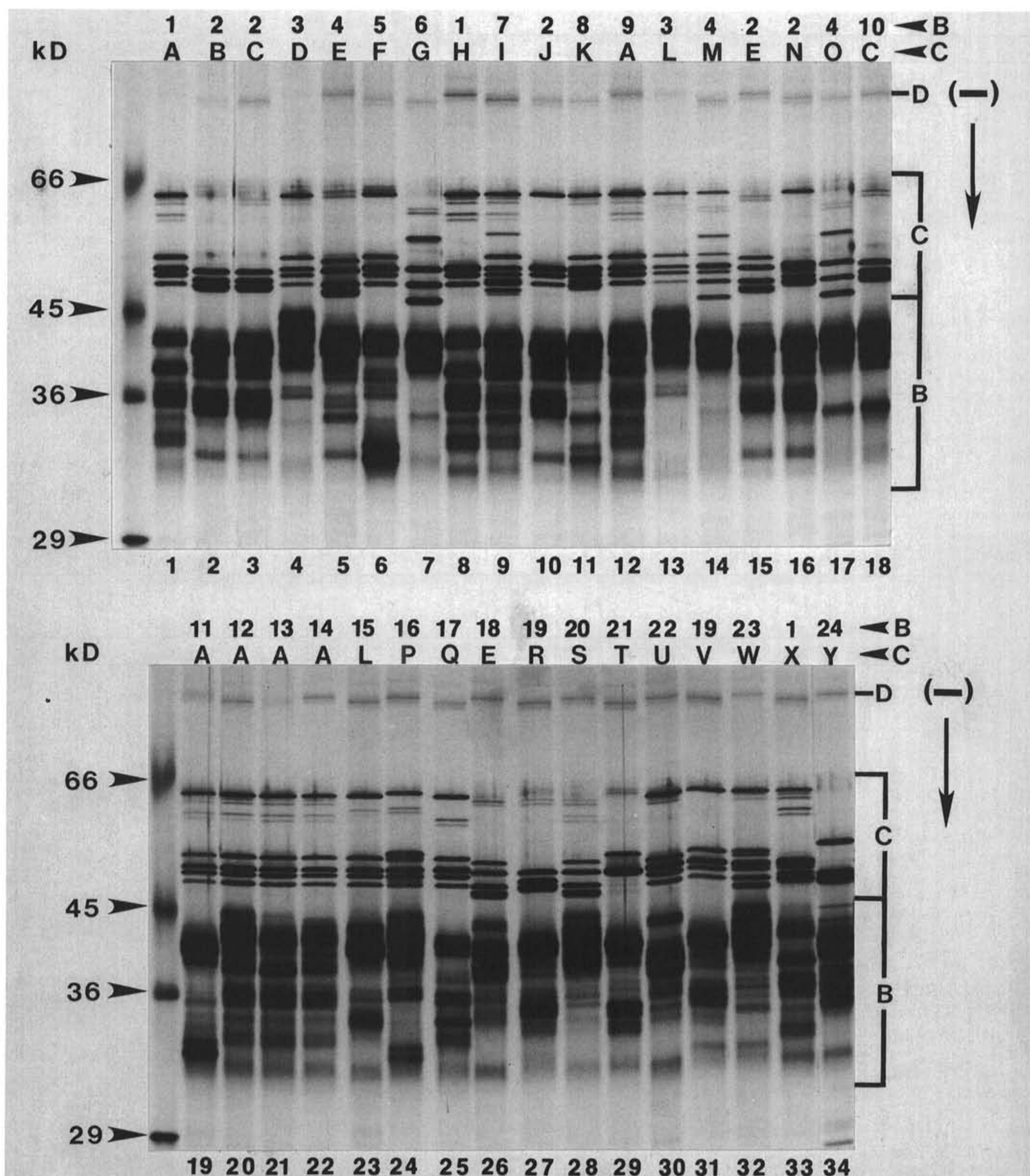

Fig. 2. The 34 different hordein electrophoretic patterns found during this study. Hordeins were extracted by the rapid sample preparation method. Numbers above the figure designate the B hordein band patterns, and letters designate the C hordein band patterns of each lane (Table I). Numbers below each lane designate the cultivar: **1,** Robust; **2,** Betzes; **3,** Klages; **4,** Beacon; **5,** Arivat; **6,** Moravian III; **7,** Schuyler; **8,** Advance; **9,** Primus; **10,** Bowman; **11,** Andre; **12,** Azure; **13,** Bedford; **14,** Boyer; **15,** Gus; **16,** Hector; **17,** Kamiak; **18,** Kimberly; **19,** Kombar; **20,** Larker; **21,** Manker; **22,** ND7309; **23,** Nordic; **24,** Otis; **25,** Pirouette; **26,** Prato; **27,** Premier; **28,** Steptoe; **29,** Summit; **30,** Sunbar 401; **31,** Triumph; **32,** Unitan; **33,** Vanguard; and **34,** Westbred 501. The numbers at the left are molecular weight designations corresponding to the electrophoretic positions of bovine serum albumin (66,000), egg ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), and carbonic anhydrase (29,000).

effectively with 55% isopropanol containing 2% 2-mercaptoethanol, in a sonication bath at 60°C, as they were by more rigorous methods. We used these conditions for this study and obtained reproducible results.

Of the three sample preparation methods (salt precipitation, dialysis precipitation, and rapid preparation), the rapid preparation method gave extracts that yielded the most reproducible banding patterns. A few minor C hordein bands, present in most cultivars examined, consistently stained more heavily (Fig. 1c) when proteins were prepared by this method than when they were prepared by either the salt or dialysis precipitation methods (Fig. 1d and g). These bands were reliable for cultivar identification only when the rapid preparation method was used. These minor bands were observed in each of two Morex samples, each grown in three locations (North Dakota or Minnesota, Wisconsin, and in a greenhouse), when the rapid preparation method was used. Similar bands, if present (Fig. 2), were observed in both samples of the other cultivars. Smith and Payne (1984) reported the presence of similar bands in barleys with an extraction solution that dissolved nearly all endosperm proteins.

Hordein preparations obtained by precipitation with aqueous NaCl also gave consistent banding patterns (Fig. 1d), aside from the minor bands mentioned above, and this method was preferred over dialysis precipitation, because dialysis precipitation took longer and several of the C hordeins remained in the supernatant (Fig. 1f) when the precipitated protein was collected by centrifugation. These proteins became soluble in water whether the sample was exhaustively dialyzed before or after alkylation, but could be avoided by using less extensive dialysis. This may indicate that some dialyzable component(s) of the extraction mixture was (were) responsible for the insolubility of these polypeptides in aqueous solution. Baxter (1976) suggested that the insolubility of hordein in water was caused in large part by lipids and polyphenols. Extraction of lipids with acetone prior to hordein extraction and/or the inclusion of polyvinyl pyrollidone to bind polyphenols resulted in two hordein fractions from barley meal samples, one soluble and one insoluble. An alternative explanation for the observation that some C hordeins were water soluble could be that they were, in fact, not true hordeins. Montembault et al (1983) suggested that the C hordein group can be further divided into C_1 and C_2 components, with the C_2 proteins possibly being albumins or globulins. It may be that the soluble C hordeins described here correspond to this C_2 fraction. Whatever the reason for the observed solubility of this polypeptide fraction, we suggest that, for quantitative protein yields, precipitation by dialysis should be avoided unless the total dialysate is recovered by lyophilization rather than centrifugation.

The reduced thiol groups of proteins are often alkylated prior to electrophoresis to prevent them from oxidizing and forming disulfide bonds, which can cause proteins to fold or to aggregate. By preventing such bonds from forming, alkylation can increase electrophoretic resolution and change the mobilities of proteins that have sulfhydryl groups (Lane 1978). In this study, alkylation of samples before electrophoresis increased the resolution of the major B hordein bands but resulted in the loss of one or more minor B hordein bands when silver staining was used (Fig. 1d and e). There were also changes in the mobilities of some of the B hordein proteins upon alkylation. Alkylation improved the resolution of some hordein bands on CBBR stained gels (Fig. 1a and b) and resulted in an increase in staining intensities, especially of the D hordeins (Fig. 1a, arrow). Alkylation did not significantly improve the resolution or shift the mobilities of C hordein bands stained with either silver or CBBR. This might be expected, because C hordeins contain few or no sulfhydryl groups (Shewry and Miflin 1985).

Staining and Storage of Gels

Both CBBR staining methods gave equivalent results (not shown). The resolution of bands stained with silver was superior to that of those stained with CBBR (Fig. 1). This was especially true for nonalkylated samples, where more bands were evident in the patterns stained with silver (Fig. 1b vs. 1c or d). Lower amounts of protein were loaded on silver-stained gels and resulted in less band spreading and better resolution. Alkylation did not result in any further differentiation among cultivars when gels were stained with silver. Without alkylation, samples were prepared in about 35 min, about the time it took to polymerize a stacking gel. Silver staining had the additional advantage of being more sensitive. Less than 0.02% (approximately 0.3 μg) of the total extractable hordein from a kernel was needed to load a lane of the mini-slab cell. This allowed the use of only a small piece of endosperm for hordein analysis, leaving the kernel essentially intact for further studies. Identical electrophoregrams were obtained using meals from whole kernels or ground up pieces of endosperm.

The D hordeins did not consistently stain well with either silver or CBBR (Fig. 1).

Gels could be fixed indefinitely in 12% TCA, but not in the methanol, acetic acid, and water fixative. Protein bands were stable for 16 hr, but the C hordein bands diffused from gels left in the solution of methanol, acetic acid, and water for 72 hr. No diffusion of B hordeins was apparent in either fixative. TCA was therefore routinely used for fixation.

Once dried, the stained gels were stable and suitable for

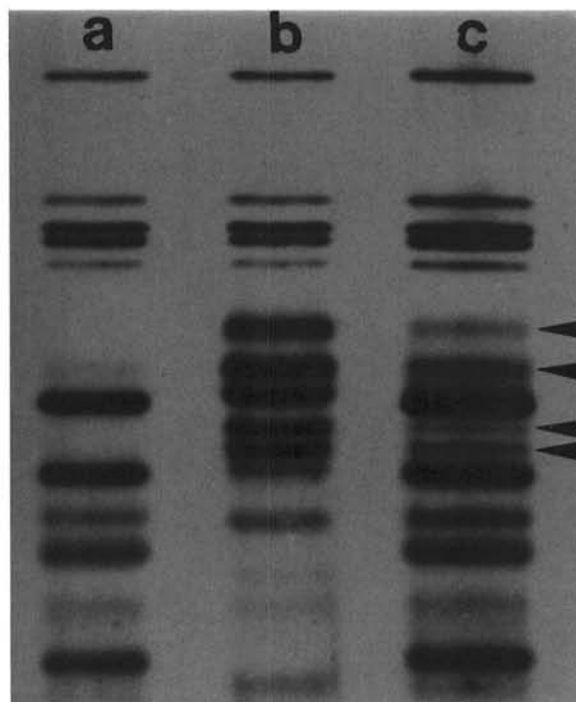

Fig. 3. Alkylated hordeins from whole meal extracts of **a**, Morex; **b**, Bedford; and **c**, a 1:9 mixture of Bedford and Morex. The arrows indicate bands contributed by Bedford. Samples were run from the cathode (top) to the anode.

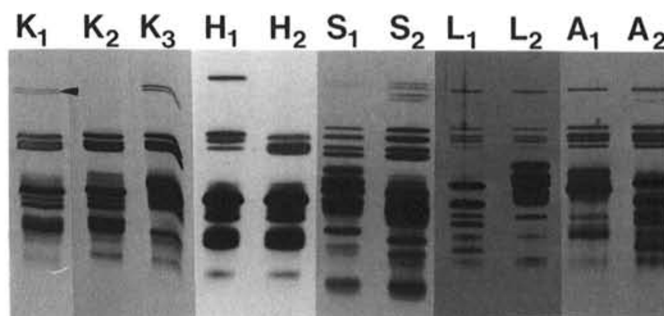

Fig. 4. Banding patterns of hordeins prepared (rapid preparation method) from single seeds of cultivars yielding more than one pattern. Letters **K**, **H**, **S**, **L**, and **A** stand for Klages, Hector, Steptoe, Larker, and Azure, respectively. Subscripts identify the different patterns found in seeds of a given cultivar. The arrow indicates the two bands that distinguish K_1 from K_2. Samples were run from the cathode (top) to the anode.

photographing, as evidenced by Figures 3 and 4, both of which are photographs of dried gels.

Hordein samples stored in SDS sample buffer degraded upon prolonged storage. After four to six weeks of storage at −20° C, the bands began to show trailing. Storage at −80° C increased their stability by about another four weeks. However, the trailing material did not obscure any bands, and six-month-old samples still gave electrophoresis patterns that were characteristic of the different cultivars.

TABLE I
Barley Cultivars Analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Cultivar	Seed Characters[a]	Pattern[b]
Bonanza	BLS 6	1A
Bumper	WLR 6	1A
Glenn[c]	WLR 6	1A
Hazen[c]	WLS-S 6	1A
M47	WSR 6	1A
Morex[c]	WSS 6	1A
Robust[c]	WSS 6	1A
Betzes	WLR 2	2B
Clark[c]	WLR 2	2B
Lamont	WLR 2	2B
Lewis	WLR 2	2B
Shabet	WLR 2	2B
Klages[c]	WLR 2	2C
Kris	WLR 2	2C
Beacon	WSR 6	3D
Karl	WSR 6	3D
Karla	WLS 6	3D
Arivat	WLS-S 6	4E
Briggs	WLS-S 6	4E
Gustoe	BLR 6	4E
Lud[c]	WSR 2	5F
Menuet	WSR 2	5F
Moravian III[c]	WLR 2	5F
Hesk	WLR 6	6G
Schuyler	WSR 6	6G
Advance	WSR 6	1H
Piroline[c]	WSR 2	1H
Primus	WLS 6	7I
Primus II	WLS 6	7I
Bowman	WLS 2	2J
Compana	WLS-S 2	2J
Andre	WSR 2	8K
Azure[c]	BLS 6	9A
Bedford	WSS 6	3L
Boyer	WLR 6	4M
Gus	BLS-S 6	2E
Hector[c]	WLR 2	2N
Kamiak	L-BSR 6	4O
Kimberly	WLR 2	10C
Kombar	WSR 6	11A
Larker[c]	WLS-S 6	12A
Manker	WSR 6	13A
ND7309	WSS 6	14A
Nordic	WSR 6	15L
Otis	WLS 2	16P
Pirouette	...	17Q
Prato	WLS-S 6	18E
Premiere	WLR 2	19R
Steptoe[c]	WLR 6	20S
Summit	WSR 2	21T
Sunbar 401	...	22U
Triumph	WLR 2	19V
Unitan	WLS-S 6	23W
Vanguard	WSR 2	1X
Westbred 501	WLS-S 6	24Y

[a] Letters refer to aleurone color (W, white; B, blue; L-B, light blue), rachilla hair length (L, long; S, short), and awn texture (R, rough; S, smooth; S-S, semi-smooth), respectively. Numbers represent rows per head.

[b] Numbers and letters designate the B and C hordein banding patterns, respectively.

[c] Single seed analysis performed.

Electrophoresis

The A hordeins were not useful for cultivar identification (Shewry et al 1978a, Montembault et al 1983) and did not stain well with silver. They were routinely electrophoresed off the bottom of the gel.

The electrophoretic patterns obtained with the model 220 electrophoretic apparatus were nearly identical to those obtained with the mini-slab apparatus. Some of the minor B hordein bands did not show up as well on the smaller gels, but cultivar distinctions were still obvious. A higher concentration of acrylamide monomer (15%) in the separating gel afforded the best resolution on the mini-slab, whereas a 13% gel gave the best results in the model 220 slab cell. Resolution of the C hordeins was often superior on the smaller gels. The short run time of the mini-slab cell (1 vs. 5 hr) allowed 50% more samples to be run per day, and the smaller gel used considerably smaller amounts of reagents per sample run. The use of a dual mini-slab cell now available (Hoefer Scientific Instruments, San Francisco, CA) allows twice as many samples to be run in a given time period.

Cultivar Identification

Analyses of hordeins from whole-meal samples from 55 cultivars (Table I) yielded 34 different hordein patterns (Fig. 2), 24 of which were unique to a single cultivar. Each C hordein pattern was given a letter (A–Y), and the different B hordein banding patterns were labeled numerically (1–24) as was done by Shewry et al (1978b). Our designations do not correspond directly with the patterns of others (Shewry et al 1978b, Curtis and Chadwick 1983). The C hordein patterns A, D, F, L, Q, and V differed in minor bands that were only consistently present when samples were prepared by the rapid method. This was also true for the patterns designated B, C, and R, as well as three other "pairs" of patterns: E and S, H and J, and N and X. Over half of the cultivars did not have unique patterns. Members of certain pattern groups (1A, 3D, 4E, 5F, 6G, and 2J) may be differentiated by their seed characteristics (Table I).

Results of this study were similar to those of Gebre et al (1986) who used an acidic PAGE system. Our system showed differences between Bedford and Karl, Azure and Nordic, and Manker and Bonanza (Table I), which the acidic system did not. Gebre et al (1986) however, were able to distinguish between Beacon and Karl, whereas we could not.

In a blind test, in which seed from 10 cultivars yielding different patterns was extracted and analyzed, each cultivar was successfully categorized as to its pattern designation (Table I) by comparison with a library of banding patterns obtained from whole-meal extracts of the original 55 cultivars analyzed.

Figure 3 shows that this technique detected a 10% admixture of two cultivars if they had sufficiently different electrophoretic patterns. Identifying cultivars comprising a mixture, however, would require the analysis of a number of individual seeds. The actual composition percentages of each of two or more barley cultivars comprising such a mixture would be difficult to determine from a whole meal sample and would require the analysis of many single seeds.

Analyses of individual seeds from European barley cultivars shows that some of them contain seeds that yield different electrophoretic patterns (biotypes) (Shewry et al 1978b, Kapala 1981, Curtis and Chadwick 1983, Smith and Payne 1984). For this study, 26 individual seeds of every barley cultivar grown on 2% or more of the planted acreage in the western or midwestern United States in 1985, as reported by the American Malting Barley Association (Table I), were extracted and analyzed. If a cultivar yielded more than one biotype upon the initial analysis of 26 seeds, the result was confirmed by a second analysis of breeder's seed from another source, except in the case of Piroline. Figure 4 shows the different patterns obtained from those cultivars that contained more than one biotype. Larker and Steptoe both contained seeds yielding two patterns, each of which constituted approximately

50% of the sample. A 1:2 ratio was found for the A_1 and A_2 patterns, respectively, of Azure. The H_1 pattern was found in about 20% of the seed in Hector samples; the rest showed the H_2 pattern. Finally, Klages had three distinct band patterns, with approximately 75% of the seed in a sample yielding the K_2 pattern and about 16% of seed showing the K_3 banding pattern. The third pattern found in Klages seed (K_1) was present in approximately 9% of the seed and differed from the major (K_2) pattern only by the two bands designated by the arrow in Figure 4. These two bands were also present in K_3. The percentages listed here are based on relatively small sample sizes (26 seeds) and are likely to show considerable variation in different samples.

CONCLUSIONS

The results of this study indicate that SDS-PAGE patterns of hordein proteins are useful for the identification of many U.S. barley cultivars. The use of a sensitive silver stain allowed the loading of low protein amounts, which led to increased band resolution and made alkylation unnecessary for most routine applications. Alkylation may be useful when many closely spaced bands are present, as in the case of a mixture of cultivars that have different banding patterns. Electrophoresis with a mini-slab cell allowed the use of smaller amounts of expensive reagents and shortened the total analysis time to approximately 5 hr. Work to further differentiate among the individual members of the groups of cultivars indistinguishable by SDS-PAGE is in progress.

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