High-Performance Liquid Chromatographic and Electrophoretic Analysis of Hordein During Malting for Two Barley Varieties of Contrasting Malting Quality[1]

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ABSTRACT

The hordeins of two barley cultivars with contrasting malting quality were studied during malting. Hordeins were sequentially extracted with 50% 1-propanol, 50% 1-propanol containing 1% dithiothreitol, and 8*M* urea containing 1% dithiothreitol. The protein composition of these extracts then was analyzed qualitatively and quantitatively by sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis and reversed-phase high-performance liquid chromatography. Substantial differences in the distribution of protein among the B, C, and D hordeins of the sound grain of these varieties were noted. The rate of decrease of total hordein during malting differed between varieties, and the rate of decrease of the individual groups of hordein also differed with the D hordein being degraded most rapidly. The relative proportion of total hordein extracted by each solvent differed between varieties as did the relative proportions of the individual groups of hordeins. Ratios of B hordein extracted in the different solvents were determined and they were substantially different between varieties. The D hordein was extracted primarily in the urea solvent. A qualitative and quantitative comparison of the hordein composition of this extract to that of the sodium dodecyl sulfate gel protein showed similarities. Differences in the amount of hordein extracted by a hot water extraction procedure also were observed between varieties.

Hordeins, which constitute about 30–50% of the total barley protein (Shewry et al 1978b, Baxter 1980), represent an important determinant of end-use quality. Studies indicate that the degree of hordein degradation during malting can influence the availability of starch granules to attack by α-amylase during mashing (Slack et al 1979). In addition, the physico-chemical nature of hordeins probably affects the rate of starch extract release during malting (Palmer 1983). It has been reported that insufficient degradation of hordein during malting can lead to a decrease in extract yield, filtration problems, and adverse effects in the final product by introducing off-flavors and haze formation (Baxter and Wainwright 1979). Therefore, this protein fraction appears to play an integral role in determining quality and profitability, and for this reason considerable effort has been directed towards a more complete understanding of the hordeins.

Hordein is found in the barley endosperm as a heterogenous mixture of polypeptides. Hordeins have been categorized into three groups called the B, C, and D hordeins based on migration during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Miflin and Shewry 1977, Field et al 1982). These groups exhibit apparent molecular weights in the range of 35,000–46,000; 55,000–72,000, and greater than 100,000, respectively, by SDS-

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PAGE. They also differ in amino acid composition with the C hordeins being notably sulfur poor whereas the B hordeins as well as D hordeins are comparatively sulfur rich (Bright and Shewry 1983). The presence of cysteine in the B and D hordeins enables these components to form disulfide-linked aggregates. In order to efficiently extract these proteins from barley, a reducing agent must be included in a solvent such as 50% 1-propanol at elevated temperatures of about 60° C (Shewry et al 1980b). Baxter (1981) has suggested that the extent to which hordein is cross-linked will have some bearing on malting and brewing quality. Comparison of the relative amounts of hordein extracted in the presence and absence of reducing agent has been carried out to determine the relationship between disulfide bonding and malting quality. The results suggest that the relative amounts of hordein extracted under reducing and nonreducing conditions are related at least in part to malting quality (Baxter 1981, Miflin et al 1983). Baxter and Wainwright (1979), after studying a small number of barley varieties by PAGE at acidic pH, suggested that the fast moving B hordeins are linked to malting quality. Subsequent studies on more barley varieties (Riggs et al 1983) and with different electrophoretic procedures (Shewry et al 1980a) showed that at best only a loose correlation exists between malting quality and electrophoretic hordein banding patterns. Smith and Simpson (1983) also looked at banding patterns of barleys as compared to their malts. The results of this study suggest that failure to digest a high-molecular-weight hordein component present in the SDS gel fraction could confer poor malting quality. Further work by Smith and Lister (1983) showed that SDS gel nitrogen could be used to predict malting quality. They also confirmed that the high-molecular-weight protein component of the gel N is D hordein, and they suggested that it could be the determining factor in the formation of aggregated protein.

In this study, two complementary protein separatory techniques were used to resolve hordein proteins. The first was SDS gradient polyacrylamide gel electrophoresis (SDSGPAGE), which separates on the basis of molecular size, and the second was reversed-phase high-performance liquid chromatography (RP-HPLC), which separates on the basis of surface hydrophobicity and is amenable to quantitation. The latter technique has been used successfully for barley and malt cultivar identification purposes (Marchylo and Kruger 1984, 1985). Two barley cultivars of contrasting malting quality were malted, and samples selected at intervals during this process were extracted sequentially. These extracts were analyzed by the techniques described above to determine if there are qualitative or quantitative differences in the hordeins that might be related to quality.

MATERIALS AND METHODS

Chemicals and Reagents

Reagent-grade chemicals were used throughout this study. Acetonitrile (HPLC grade) with an ultraviolet cutoff wavelength of 190 nm was obtained from Fisher Scientific (Fair Lawn, NJ). Sequanol-grade trifluoroacetic acid and 1-propanol were obtained from Pierce Chemical Co. (Rockford, IL). Deionized water was purified using a Millipore Super Q system (Millipore Corp., Bedford, MA) as described previously (Marchylo and Kruger 1984).

TABLE I
Pilot Malting Data[a]

Analysis	Bonanza	Diamond
Protein, %	13.7	13.4
Extract, %	77.6	73.6
Fine/Coarse extract, %	4.8	13.3
Soluble/Total protein, %	39.3	28.5
Diastatic Power, ° L	141	89
α-Amylase, DU	46.9	28.2

[a] Data provided by D. E. LaBerge.

Barley Samples

A malting quality six-rowed barley cultivar, Bonanza, and a newly licensed six-rowed feed cultivar, Diamond (Kaufmann and Kibite 1985), were used in this study. Bonanza barley was grown at Three Hills, Alberta, as part of the 1982 Collaborative Plant Breeders Program. Diamond barley was grown during 1982 at the Agriculture Canada Research Station in Regina, Saskatchewan. Malting quality differences between these cultivars, as indicated by analysis of pilot malts, are shown in Table I.

Malted Barley Samples

Malting was carried out using a Seeger micromalting system. The malting procedure consisted of steeping at 12° C for two days, germination for five days at 13° C, and kilning for two days to a final temperature of 85° C to produce malts with approximately 4% moisture. For each variety, eight samples of 500 g (dry basis) were placed in steep tanks simultaneously. Samples then were withdrawn after one and two days steeping, one to five days germination, and two days kilning. Subsequently, all samples with the exception of the kilned samples were freeze-dried.

Extraction Procedure

The malted barley samples and a sound grain sample for each variety were ground in a Udy cyclone sample mill equipped with a 1-mm sieve. Protein contents (% N × 6.25) were determined by the Kjeldahl method as modified by Williams (1973) using a 2-g sample. Moistures were determined by the standard AACC procedure (1 hr, 130° C). Two-gram samples of ground grain (as is) were extracted sequentially at 60° C for 30 min with vortexing at 10-min intervals, followed by centrifugation (75,000 × *g*, 15 min) using 12 ml of the following extracting solutions: a) 0.5*M* sodium chloride (two times), b) water wash, c) 50% 1-propanol (two times), and d) 50% 1-propanol containing 1% dithiothreitol (DTT). A final extraction (e) using 12 ml of 8*M* urea containing 1% DTT was carried out overnight. For simplicity, the proteins extracted in each solvent were called a) salt solubles, c) propanol solubles, d) propanol-DTT solubles, and e) urea-DTT solubles.

The water wash was inserted after the salt extraction to remove traces of salt in the residue. Fifty percent 1-propanol and a temperature of 60° C were used for optimum extraction efficiency of the hordeins (Shewry et al 1980b). Addition of reducing agent was required to extract hordein present as disulfide-linked aggregrates (Shewry 1978a, Miflin et al 1983). One percent DTT was used because it is an efficient reducing agent and is preferable for RP-HPLC analysis (Marchylo and Kruger 1984).

With the exception of the urea extracts and the water wash, protein contents of supernatants were determined by the modified Kjeldahl procedure (Williams 1973) on 9-ml aliquots that were evaporated first to dryness. Protein contents of urea-DTT extracts were estimated by RP-HPLC on the basis of their total protein peak areas as compared to the relationship between the total protein peak areas and Kjeldahl protein content of the propanol-DTT extracts. An extract of "total" hordein also was prepared for comparative purposes by a salt extraction followed by a water wash and then extraction with 50% 1-propanol containing 1% DTT. Extraction conditions were comparable to those just described. Excluding the water wash, 1-ml aliquots of supernatants were passed through 0.45 μ type HV filters (Millipore Ltd., Mississauga, Ontario) prior to RP-HPLC analysis of 50-μl injections. All subsequent extracts were filtered in this manner. A portion of the propanol extracts was reduced by mixing 100 μl of supernatant with an equal volume of 50% 1-propanol containing 2% DTT followed by heating at 60° C for 30 min. The proteins present in this extract were called the propanol solubles (reduced). Injection volumes of 100 μl were used for these samples to compensate for the twofold dilution during reduction.

Extraction of SDS Gel Protein

The SDS gel protein fraction was prepared from sound grain and five-day germinated malts of Bonanza and Diamond barley as described by Smith and Simpson (1983). Hordeins were extracted from the gel with 0.6 ml of 50% 1-propanol containing 1% DTT at

60° C for 1 hr followed by centrifugation for 10 min at 8,800 × *g*. Aliquots of 15 μl were injected for RP-HPLC analysis.

Hot Water Extract

The hot water extract was prepared using a modified Institute of Brewing procedure (Institute of Brewing 1977). Modifications included extraction at 70° C and filtration at 30° C. An injection volume of 100 μl was employed for RP-HPLC analysis.

RP-HPLC

A Waters HPLC and Waters 840 data and chromatography control station (Waters Associates, Inc., Milford, MA) was used as described previously (Marchylo and Kruger 1985). A 10 × 0.46 cm MPLC Aquapore RP-300 RP-HPLC column (particle size 10 μm pore size 300 Å, C_8 support; Brownlee Laboratories, Santa Clara, CA) preceeded by a 1.5 × 0.32 cm MPLC New Guard column (particle size 7 μm, pore size 300Å, C_8 support) was used throughout this study. A 0.5-μm precolumn filter (Upchurch Scientific, Oak Harbor, WA) was inserted before these columns and filters were changed daily. Column temperature was maintained at 31° C by a temperature controller (Eldex Laboratories, Menlo Park, CA). Solvents A and B consisted of water and acetonitrile, respectively, each containing 0.1% trifluoroacetic acid. The solvents were sparged continuously with helium to prevent baseline drift. Except for the analysis of the SDS gel protein, proteins were eluted by a complex gradient controlled by the Waters 840. The gradient consisted of a combination of linear and concave gradients as indicated in Table II. A total run time of 90 min including 9 min for equilibration at a flow rate of 1 ml/min was used for each analysis. SDS gel protein was analyzed using a linear gradient as described previously (Marchylo and Kruger 1985). Columns were cleaned regularly, using a linear gradient extending from 20–80% acetonitrile to remove small amounts of adsorbed proteins. Eluted proteins were monitored at 210 nm. Absorbances were monitored in volts, with 2V equivalent to two absorbance units full scale. Chromatograms were integrated by the computerized Waters 840 system. Where required, individual peaks within a chromatogram were integrated by using the scanner mode of the Waters 840. This enabled the operator to choose appropriate baselines and to treat chromatograms on an individual basis. Plots of chromatograms were prepared also in the scanner mode and were attenuated to provide optimum visualization of protein peaks.

SDSGPAGE

Aliquots of supernatants (100 μl) from the sequential extraction procedure were mixed with an equal volume of 0.5*M* iodoacetamide to alkylate the reduced proteins and prevent reoxidation. The mixture was heated for 15 min at 60° C and then was mixed with an equal volume of a pH 8.6 buffer solution containing 2% SDS. This solution subsequently was heated at 60° C for 15 min. One microliter of each sample mixture was loaded per sample well prior to electrophoresis. Total hordein extracts also were prepared at each stage of malting. This was done by first extracting 2-g samples with a 0.5*M* sodium chloride solution followed by extraction with 50% 1-propanol containing 1% DTT as described in the extraction procedure. Prior to electrophoresis, 100 μl aliquots of the extracts were heated as described above. Gels were calibrated for molecular weight determinations by analyzing a mixture of standard proteins included in an electrophoretic low-molecular-weight calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden). Of the standard proteins present in the kit, phosphorylase-b (mol wt 94,000), bovine serum albumin (mol wt 67,000), ovalbumin (mol wt 43,000), and carbonic anhydrase (mol wt 30,000) were used for preparation of calibration curves. Proteins were resolved by SDSGPAGE (11–17% linear acrylamide gradient) using a Bio-Rad Protean vertical electrophoresis cell. Wells were formed in a 4% stacking gel that was removed before staining. Electrophoresis was carried out for 2.5 hr at 10° C at a constant power of 40 W. Following electrophoresis, proteins were stained by a silver-based color stain (Marchylo, *unpublished data*).

TABLE II
Complex Gradient for the Separation of Hordeins by Reversed-Phase High-Performance Liquid Chromatography

Time (min)	Flow (ml/min)	Gradient	%A (Water)	%B (Acetonitrile)
Initial conditions	1.00	...	80	20
3.00	1.00	1[a]	80	20
23.00	1.00	6[b]	68	32
36.00	1.00	1	68	32
56.00	1.00	7[c]	61	39
81.00	1.00	8[c]	51	49
83.00	1.00	6	80	20
90.00	1.00	1	80	20

[a] 1 indicates an isocratic gradient.
[b] 6 represents a linear gradient.
[c] 7 and 8 are concave gradients with 8 being the most concave.

Identification of D Hordein

Sound Bonanza barley was extracted sequentially and five RP-HPLC analyses of urea-DTT extracts were carried out as described with the exception that 100 μl of extract was injected per run. Fractions (2 ml) corresponding to the peak eluting at about 31 min (Fig. 6A, urea-DTT soluble) were collected during each run. Acetonitrile then was evaporated from the resultant 10 ml solution by flushing with a stream of nitrogen. The remaining liquid was concentrated to 1 ml using an Amicon Ultrafiltration cell with a Diaflo ultrafiltration UM10 membrane (Amicon, Lexington, MA) and freeze-dried. The freeze-dried protein was redissolved in 100 μl of 50% 1-propanol containing 1% DTT. This concentrate was subsequently divided into 50 μl portions with one portion being reanalyzed by RP-HPLC whereas the other was analyzed by SDSGPAGE as described previously.

RESULTS

The malting cultivar Bonanza and the feed cultivar Diamond exhibited similar protein contents (dry basis) of 13.5% and 14.2%, respectively. Albumins, globulins, and nonprotein nitrogen were extracted first with 0.5*M* NaCl to prevent contamination of the hordein fractions. The salt solubles increased from 28.5% and 24.9% of the total seed nitrogen in the sound grain samples to 50.3% and 42.6% in the five-day germinated samples for Bonanza and Diamond, respectively. Total hordein in each sample was

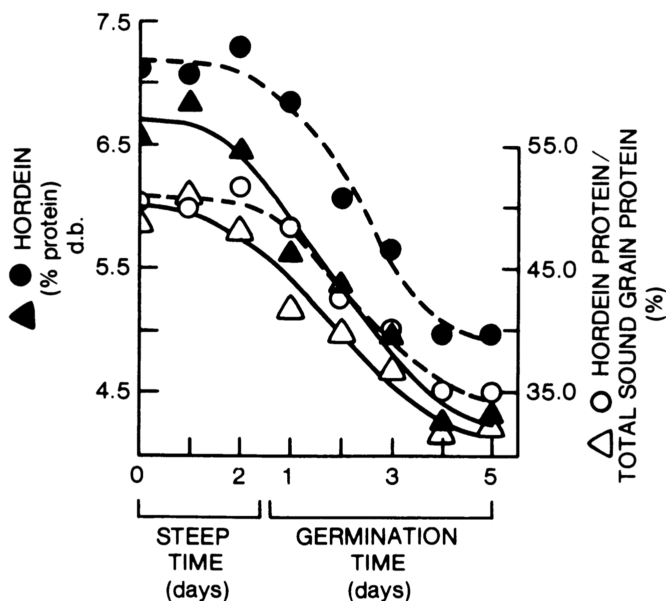


Fig. 1. Decrease in hordein protein content in the six-rowed varieties Bonanza (malting, Δ, Δ) and Diamond (feed, o, o) during malting.

determined by summation of the protein extracted in the sequential extraction schemes, excluding the salt solubles. Changes during malting in hordein protein content on an absolute basis and relative to the total sound grain protein then was plotted as shown in Figure 1. Hordein comprised about 50% of the total sound grain protein for both cultivars. In Diamond, this represented 7.1% protein as compared to 6.6% in Bonanza. After five days of germination, the hordein content decreased (Fig. 1) to 5.0% (36% of sound grain protein) and 4.3% (32% of sound grain protein) for Diamond and Bonanza, respectively.

Qualitative Analysis of Hordeins by SDSGPAGE

SDSGPAGE was used to determine the hordein banding patterns for a total extract at each stage of malting. The protein patterns did not change qualitatively during malting for either cultivar, which is consistent with previous reports (Shewry et al 1980a, Montembault et al 1983, Smith and Simpson 1983). Examples of the total hordein patterns are shown in Figure 2. Differences in the hordein banding patterns were present in the C and B hordeins but the D hordein patterns appeared comparable. The protein patterns in extracts from the sequential extraction procedure also were determined by SDSGPAGE.

Examples of the patterns obtained are illustrated in Figure 2 for extracts prepared from the sound grain and five-day germinated samples of each cultivar. The salt solubles also were analyzed to ensure that cross contamination of the hordein extracts did not occur (Fig. 2A, B). Comparison of the salt-soluble and the hordein patterns indicated that cross contamination was not a problem.

The propanol-soluble hordeins (Fig. 2C, sound) contained high-molecular-weight components of estimated mol wt > 250,000 for Bonanza and > 180,000 for Diamond. Trace levels of D hordein were noted for Bonanza but not Diamond. For both cultivars, the C hordeins represented the major hordein component extracted, and only small amounts of protein were stained in the B hordein region of the gels. Analysis of the second propanol extracts (Fig. 2D, sound) showed that only small amounts of the same components were extracted.

Propanol extracts then were reduced to disrupt disulfide linkages and the protein patterns were determined by SDSGPAGE. Reduction significantly changed the protein patterns (Fig. 2E, sound). The high-molecular-weight components disappeared and protein bands, essentially equivalent to the B hordein bands observed in the total extracts, appeared. The C and D hordein bands did not appear to change.

Extraction of the residue with propanol-DTT resulted in the further solubilization of D and B hordein and trace quantities of C hordein (Fig. 2F, sound) comparable to those present in the propanol-soluble (reduced) extracts. Small amounts of the same components were removed by a second extraction (Fig. 2G, sound).

Extraction of the remaining grist with urea-DTT removed primarily D and B hordeins and only trace levels of C hordein (Fig. 2H, sound). To ensure that hordeins extracted with urea-DTT did not result from incomplete extraction by propanol-DTT, a sequential extraction was carried out in which four propanol-DTT extractions were employed. The urea-DTT extract in this analysis showed comparable protein patterns (as well as RP-HPLC chromatograms), which indicated that incomplete extraction was not a problem.

Protein patterns obtained for extracts of samples of both cultivars, selected during malting as compared to the sound grain extracts, remained essentially the same on a qualitative basis. For example, protein patterns for the extracts of the five-day germinated samples are illustrated in Figure 2. However, the propanol extracts (Fig. 2C, five-day germinated) exhibited a series of weakly stained bands extending from below the C hordein region into the B hordein region of the gels. Also some of the B hordeins in the propanol-soluble (reduced) extracts of Bonanza five-day germinated and kilned samples exhibited an apparent decrease in relative intensity (Fig. 2E, five-day germinated).

Qualitative Analysis by RP-HPLC of Hordeins

Salt-soluble proteins were analyzed first to ensure that cross-contamination with hordein proteins was not a problem (Fig. 3 and 4, A-E). As shown in Figure 3A (salt solubles), the majority of salt-soluble proteins were more hydrophilic than the hordein proteins (Fig. 3A, propanol-soluble [reduced]) and were eluted within 30 min.

Further comparison of chromatograms and retention times indicated that the more hydrophobic salt-soluble protein peaks did not coincide in elution times with hordein peaks. The presence of proteins eluting in the salt-soluble region (10–20 min) of the urea-DTT chromatograms also was detected. These proteins may have represented a small amount of strongly-bound albumin and globulin proteins. However, only low levels were detected, and these peaks did not coincide and consequently interfere with the hordein protein peaks.

RP-HPLC analysis of the propanol-soluble hordeins after reduction (Figs. 3 and 4, A–E) indicated that throughout malting, two groups of protein peaks consistently were present. The first group consisted of C hordeins and the second B hordeins as noted previously (Marchylo and Kruger 1984). Analysis of the propanol-soluble hordeins also was carried out before reduction. As illustrated in Figure 5 for the sound and five-day germinated samples of both varieties, reduction of the propanol extracts resulted in a significant shift primarily in the B hordein elution profiles. In both cases, the elution profiles of the major C and B hordein peaks remained consistent during malting. However, a number of small peaks, eluting between the major C and B hordein peaks (60–65 min), appeared after two days steeping (Fig. 3B, Bonanza, propanol-soluble [reduced]) and then disappeared after five days of germination (Fig. 3E, propanol-soluble [reduced]).

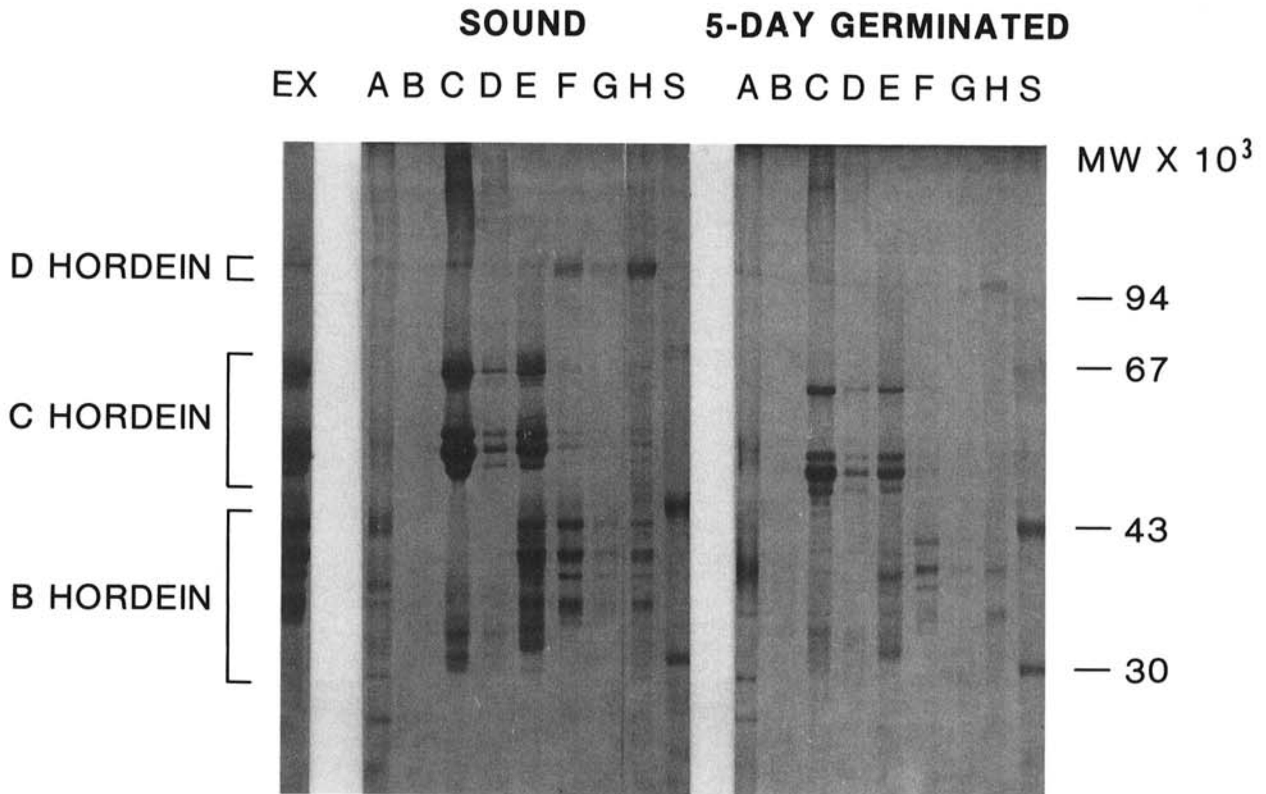
Analysis of the propanol-DTT extracts revealed that B hordein was the major storage protein present. Only trace levels of C hordeins were detected (Fig. 6A–E, Bonanza; Fig. 7A–E, Diamond). The elution profile of the B hordeins remained qualitatively the same throughout malting, for both cultivars. As seen in the elution profile for the sound grain (Figs. 6A and 7A), another relatively large peak was eluted at about 31 min. This peak represented D hordein as described in a subsequent section. It was not detectable after the second or first day of germination of Bonanza and Diamond, respectively. In addition, unidentified minor peaks were eluted before the D hordein peak but these also were lost after two and one days of germination, respectively.

Analysis of the urea-DTT extracts revealed that for both cultivars, a major hordein component was eluted after about 31 min. Identification of this component was undertaken as described in Materials and Methods. Reanalysis by RP-HPLC of the freeze-dried protein present in this peak yielded a single peak eluting at the same position as in the original run, and SDSGPAGE analysis (results not shown) yielded a protein band corresponding to the D hordein band noted in Figure 2. The apparent molecular weight of this protein was estimated at 105,000, which is consistent with previously reported estimates by SDS-PAGE for D hordein (Field et al 1982). Only small amounts of protein were detectable in the B hordein region on the Bonanza chromatogram, whereas in the Diamond chromatograms, significant quantities of protein were eluted in this region. Comparison of the Diamond urea-DTT (Fig. 7A–E) and propanol-soluble (reduced) (Fig. 4A–E) elution profiles indicated some qualitative differences in the B hordein peaks. In addition, small quantities of protein were detected in the salt-soluble region of the chromatograms of both cultivars. On a qualitative basis, chromatograms essentially remained the same throughout malting.

Quantitative Analysis of Hordeins by RP-HPLC

The computerized Waters 840 system was used to integrate chromatograms and thus provide a quantitative estimate of the relative amounts of protein contributed by individual peaks or groups of peaks comprising the B, C, and D hordeins. To ensure precise comparisons, proteins extracted by 50% 1-propanol were quantified following reduction, i.e., the propanol-soluble (reduced) chromatograms were analyzed by the Waters 840 system.

The relative rates of decrease in protein within the B, C, and D hordein groups were determined for the two cultivars. As



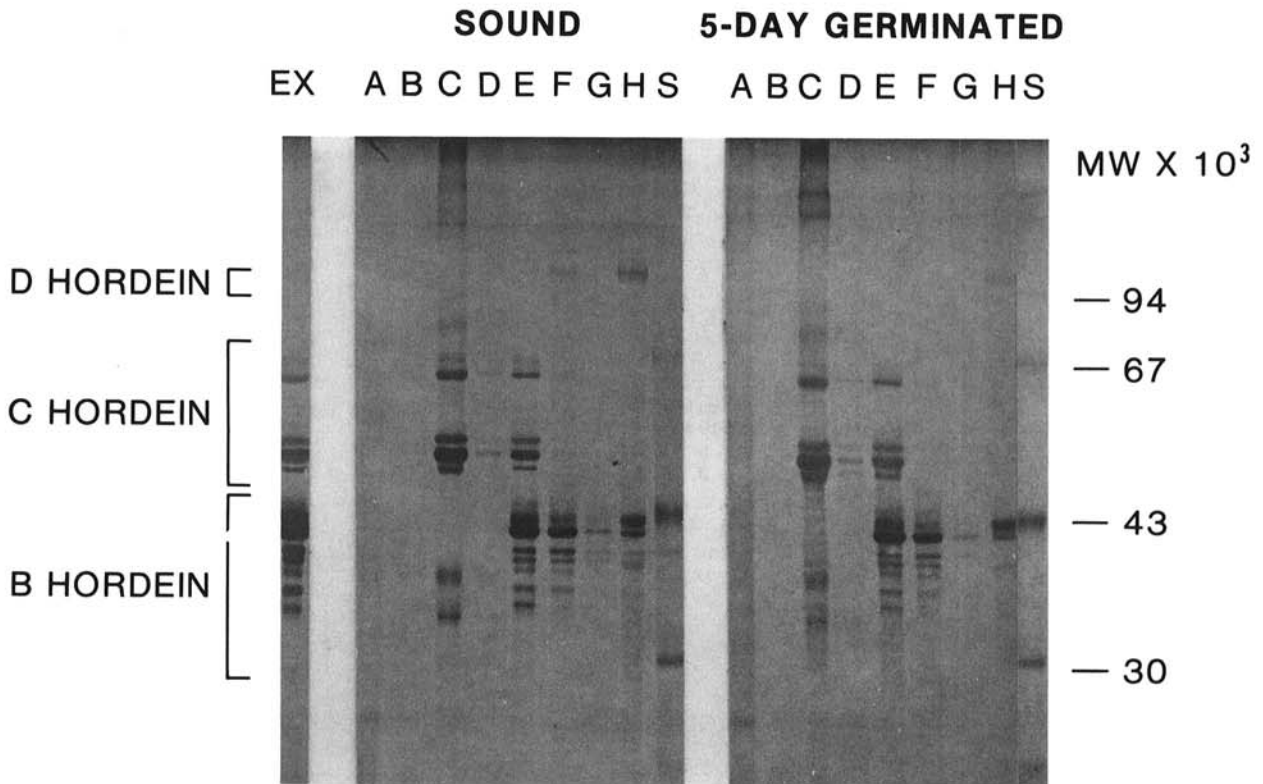


Fig. 2. Electrophoregrams prepared by sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis of barley and malt proteins. (**EX**) Total hordein extract using 50% 1-propanol + 1% dithiothreitol, following extraction with 0.5*M* NaCl. Sequential extraction of: (**A,B**) salt-soluble, first and second extraction; (**C,D**) propanol-soluble, first and second extraction; (**E**) propanol-soluble (reduced); (**F,G**) propanol-DTT-soluble, first and second extraction; (**H**) urea-DTT-soluble; (**S**) standard molecular weight protein markers: phosphorylase b (mol wt 94,000); bovine serum albumin (mol wt 67,000); ovalbumin (mol wt 43,000); and carbonic anhydrase (mol wt 30,000).

illustrated in Figure 8, the rate of decrease differed between cultivars and hordein groups. Proportions of the three groups of hordeins present in each extract and proportions of hordein extracted into each solvent relative to total hordein were calculated. Results, as shown in Tables III and IV were averaged where applicable. Significant differences between cultivars in the distribution of protein between the B, C, and D hordeins were evident (Table III). In addition, the extractability of total hordein and of each hordein group in each solvent differed appreciably between cultivars (Table IV). The relationship between individual components during malting also was studied to ascertain if preferential degradation of hordeins within a group was taking place. It was found that the ratio of the C hordein peaks U and V, for example (Fig. 3A, Bonanza), remained constant during malting at 1.30:1 ± 0.02 ($\bar{x} \pm s_{\bar{x}}$, $n = 9$), and the ratio of the B hordein peaks X and Y (Fig. 4A, Diamond) was constant at 4.00:1 ± 0.05 ($\bar{x} \pm s_{\bar{x}}$, $n = 9$).

Relative proportions of B hordein extracted into solvents employed in the sequential extraction procedure also were determined (Table V). The ratios of B hordein between solvents were consistent during malting and therefore were averaged for the nine sample extractions. In all cases, the ratios were higher significantly for Bonanza as compared to Diamond.

Analysis of Hordein from Kilned Malts

SDSGPAGE and RP-HPLC analysis of kilned malt extracts showed that qualitatively the five-day germinated malts (green malts) and kilned malts were essentially the same within each variety. Quantitative analysis of RP-HPLC elution profiles also revealed little difference (results not shown).

RP-HPLC Analysis of SDS Gel Protein

RP-HPLC analysis of protein extracted from the SDS gel fraction prepared from the sound grain of both cultivars revealed that the major hordeins present were the D and B hordeins (Fig. 9). Analysis of SDS gel protein prepared from the green malts yielded elution profiles that were qualitatively the same (results not shown). Quantitative analysis of chromatograms showed that the D:B ratio was higher for the sound grain of Bonanza as compared to Diamond and that these ratios decreased during malting (Table VI). A similar relationship was observed for the D and B hordein extracted by urea-DTT as shown by comparison in Table IV. In this instance, however, the ratio was not determined after three days of germination because low peak areas made quantitative estimates difficult. It is of note that prolonged analysis of the SDS gel proteins resulted in deterioration in resolution and shifts in retention times for the RP-HPLC columns. This may have been caused by interference from the SDS.

RP-HPLC Analysis of Hot Water Extracts

Protein peaks, as shown in Figure 10, were not sharp and were poorly resolved possibly due to interference by other components present in the extract. However, a qualitative estimate of the types of protein present could be obtained. For both varieties, proteins were eluted in the salt-soluble and B hordein regions of the chromatograms. The major difference between cultivars was the presence of a significantly higher ratio of B hordein to salt solubles in the Diamond extracts.

DISCUSSION

The six-rowed barley cultivars Bonanza and Diamond were selected for this study because they exhibited contrasting malting quality. Bonanza is a six-rowed blue aleurone barley with good malting quality and Diamond, a poor malting variety, is a yellow aleurone six-rowed barley that recently has been licensed for feed purposes (Kaufmann and Kibite 1985). Large differences in extract and soluble to total protein, for example, are observed between pilot malts of these varieties as shown in Table I. The higher levels of salt-soluble protein in the five-day germinated Bonanza malt as compared to the Diamond malt correlates with the pilot malting data.

As illustrated in Figure 1, the hordein content of Diamond, on a protein basis, was higher than Bonanza throughout malting. As a proportion of the total seed protein, the initial hordein content of both cultivars was about 50%. This is consistent with values reported in the literature (Shewry et al 1978a, 1980b). The rate of decrease in the hordein content, however, was more rapid during malting for Bonanza, which exhibited a lower proportion of hordein after the completion of malting. The two cultivars exhibited a considerable difference in the distribution of protein among the B, C, and D hordeins comprising the total hordein in the

TABLE III
Distribution of B, C, and D Hordeins in Sound Grain as a Percentage of the Total Hordein[a]

Cultivar	Hordein B	Hordein C	Hordein D
Bonanza	58.9	34.0	7.1
Diamond	70.0	24.5	7.6

[a] Average of two determinations.

TABLE IV
Extractability of Hordein into Solvents Used in Sequential Extraction Procedure

Hordein	Bonanza			Diamond		
	50% 1-Propanol	50% 1-Propanol + 1% DTT[a]	8*M* Urea + 1% DTT	50% 1-Propanol	50% 1-Propanol + 1% DTT	8*M* Urea + 1% DTT
Total hordein, % ($\bar{x} + s_{\bar{x}}$)[b]	83.5 ± 0.7	8.7 ± 0.4	7.9 ± 0.5	75.6 ± 1.1	13.7 ± 0.6	10.4 ± 0.5
$\frac{B}{\text{Total B}}$ % ($\bar{x} \pm s_{\bar{x}}$)	82.3 ± 0.5	14.6 ± 0.4	3.3 ± 0.2	73.3 ± 0.9	19.7 ± 0.7	7.0 ± 0.4
$\frac{C}{\text{Total C}}$ %	100	neg[c]	neg	100	neg	neg
$\frac{D}{\text{Total D}}$ %						
Sound grain		17.2	82.8		15.7	84.3
Germinated two days	neg	0	100	neg	0	100

[a] DTT, dithiolthreitol.
[b] $n = 9$.
[c] Negligible.

sound grain. As shown in Table III, Diamond contained considerably more B hordein relative to C hordein whereas the D hordein contents were similar. The rates of decrease of the individual groups of hordeins also were different. RP-HPLC analysis showed that the D hordein was degraded much more rapidly than the B followed by the C hordeins (Fig. 8). This confirms previous studies that B hordein is attacked more rapidly than C hordein (Baxter and Wainwright 1979) and presents additional evidence that D hordein is the most susceptible to degradation. The rate of degradation, although different between groups, remained consistent to cultivar.

Extraction with propanol removed the major proportion of

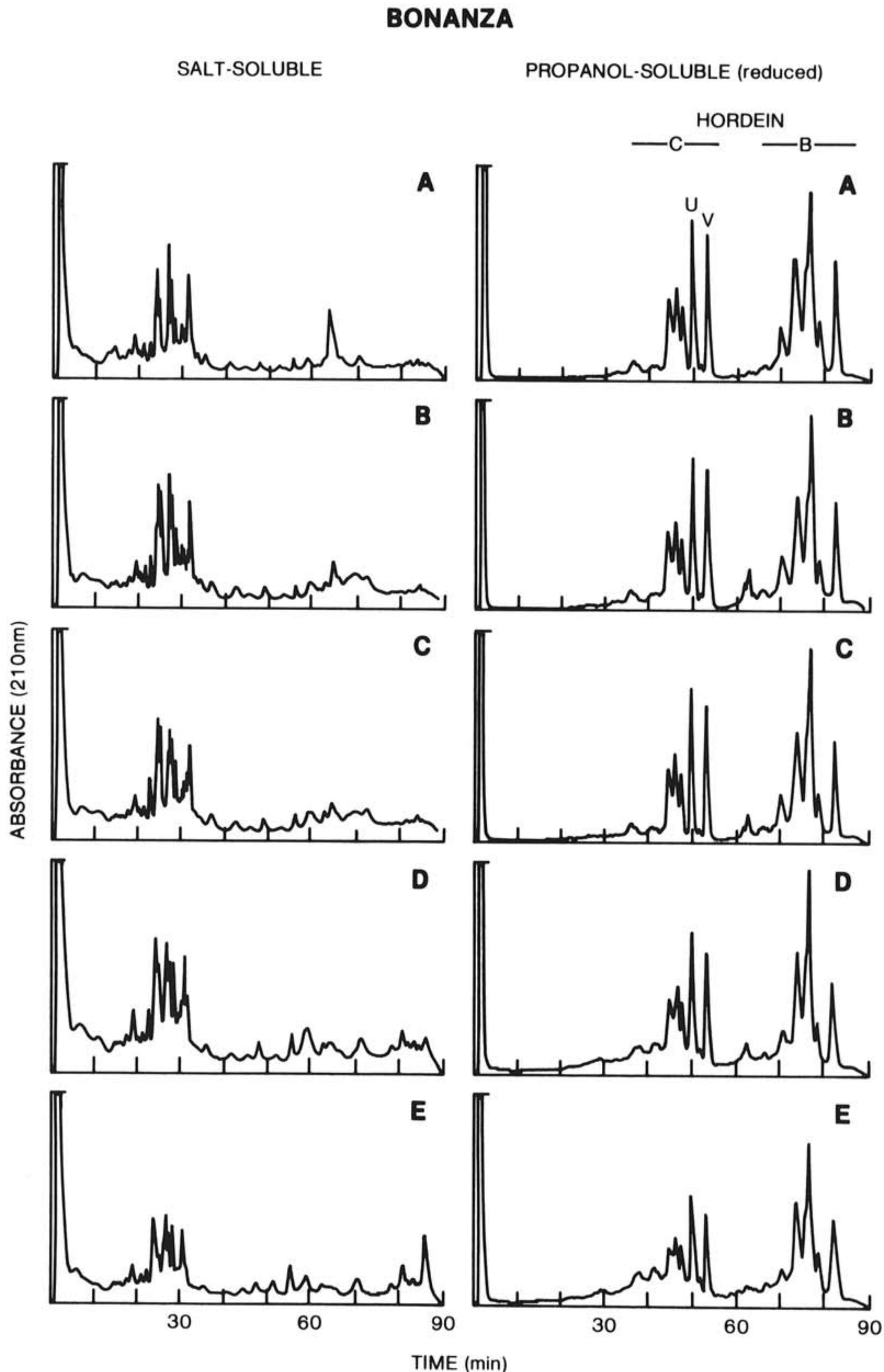


Fig. 3. Reversed-phase high-performance liquid chromatography separations of Bonanza barley and malt proteins extracted in the sequential extraction procedure with 0.5 *M* NaCl (salt-soluble) and 50% 1-propanol followed by reduction (propanol-soluble [reduced]). (**A**) sound grain, (**B**) two-day steep, (**C**) one-day germinated, (**D**) three-day germinated, and (**E**) five-day germinated.

hordein for both cultivars, although a significantly higher proportion was removed for Bonanza (Table IV). This proportion was constant during malting. SDSGPAGE and RP-HPLC analysis indicated that virtually 100% of the C hordein was present in this extract. In addition, a major proportion of the B hordeins was extracted. However, a significantly larger proportion of the Bonanza as compared to Diamond B hordein was extracted by propanol (Table IV). Negligible amounts of D hordein were removed by this solvent, in agreement with previous reports (Field et al 1983, Miflin et al 1983).

Electrophoretic analysis of the propanol-soluble and propanol-soluble (reduced) extracts showed that for all malted samples, B hordein was present primarily in the form of high-molecular-weight aggregates (Fig. 2). The banding patterns of these

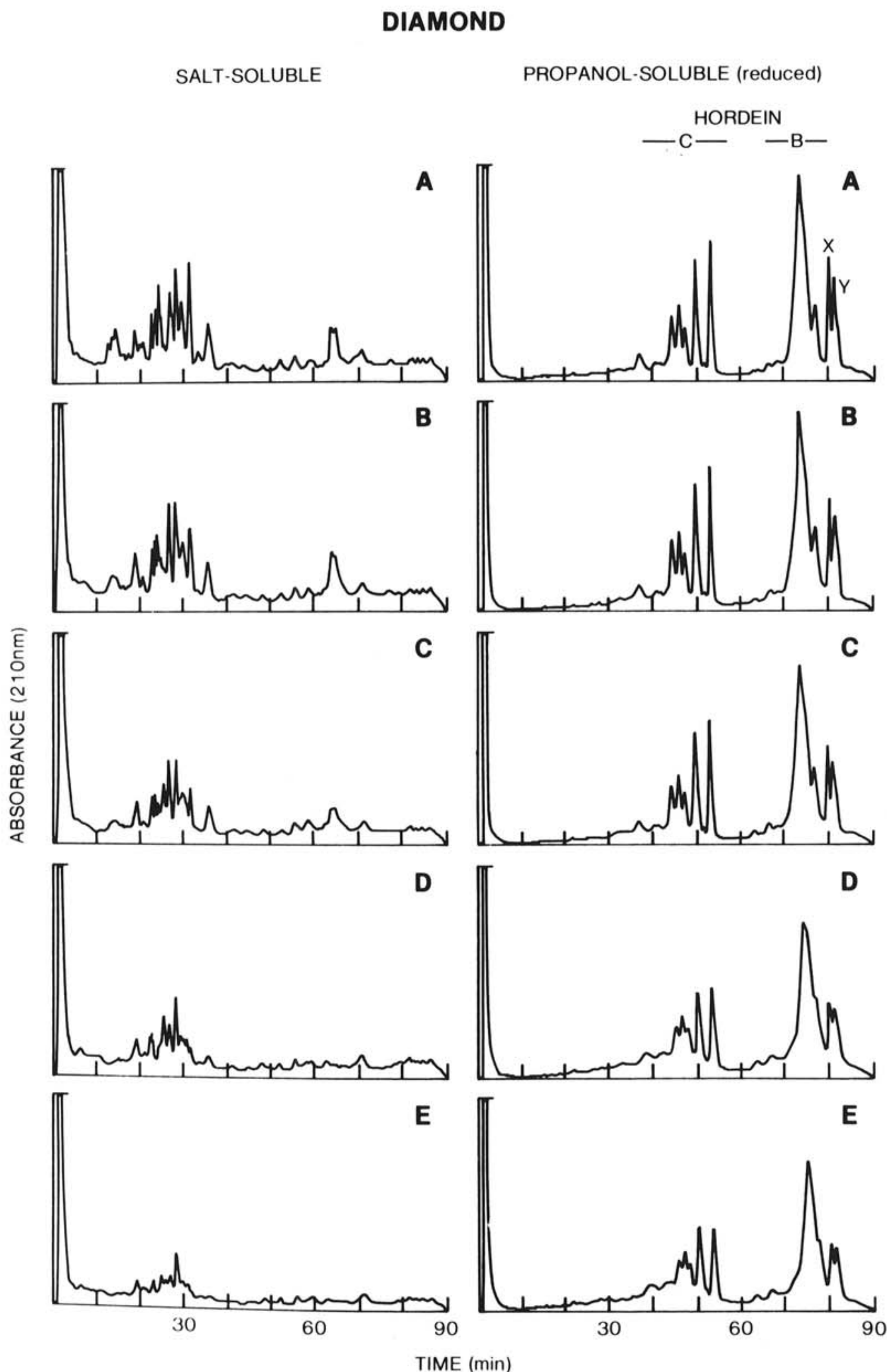


Fig. 4. Reversed-phase high-performance liquid chromatography separations of Diamond barley and malt proteins extracted in the sequential extraction procedure using 0.5*M* NaCl (salt-soluble) and 50% 1-propanol followed by reduction (propanol-soluble [reduced]). (**A**) sound grain, (**B**) two-day steep, (**C**) one-day germinated, (**D**) three-day germinated, and (**E**) five-day germinated.

components differed between the two cultivars, which would be expected because of differences in their reduced B hordein patterns. RP-HPLC analysis also showed significant differences between the reduced and unreduced extracts (Fig. 5).

Of note was the series of minor bands (SDSGPAGE) and peaks (RP-HPLC) detected between the major C and B hordein components in the malted samples. Previous studies (Smith and Simpson 1983, Marchylo and Kruger 1985) were unable to detect hordein degradation products in malt. The minor components detected in this study may represent small amounts of larger degradation products present as a result of proteolytic attack.

Subsequent extraction with propanol-DTT removed additional hordein more tightly bound within the endosperm, presumably

TABLE V
Relative Proportions of B Hordein Extracted into Solvents Employed in the Sequential Extraction Procedure

		Ratio of B Hordein Extracted in Solvent 1:2 ($\bar{x} \pm s_{\bar{x}}$)[a]	
Solvent 1	Solvent 2	Bonanza	Diamond
50% 1-propanol	50% 1-propanol + 1% DTT	5.7:1 ± 0.2	3.8:1 ± 0.2
50% 1-propanol	8M urea + 1% DTT	26.1:1 ± 1.6	10.8:1 ± 0.8
50% 1-propanol + 1% DTT	8M Urea +1% DTT	4.7:1 ± 0.4	2.9:1 ± 0.2

[a] $n = 9$.

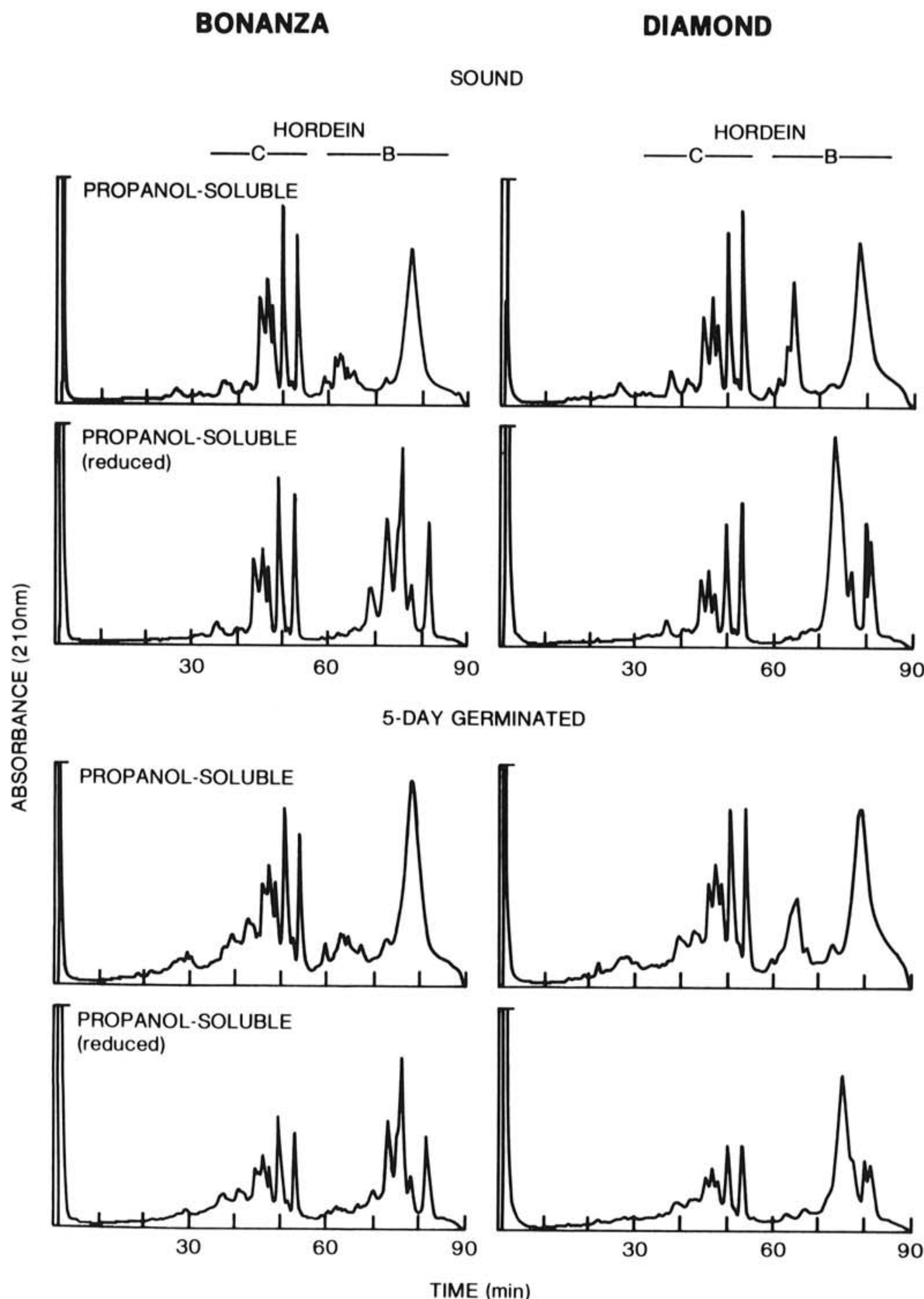


Fig. 5. Effect of reduction on the reversed-phase high-performance liquid chromatography elution profiles of the propanol-soluble hordein proteins from sound and five-day germinated Bonanza and Diamond barleys.

because of disulfide bonding among hordein molecules. With this solvent, a larger proportion of the Diamond total hordein was extracted in comparison to Bonanza (Table IV). Electrophoretic and RP-HPLC analysis revealed that the major constituent of this fraction, throughout malting, was B hordein. However, as indicated in Table IV, a larger proportion of the Diamond as compared to Bonanza B hordein was extracted in the propanol-DTT. Qualitative analysis by SDSGPAGE and RP-HPLC also indicated that this solvent differentially extracted certain B hordeins in Diamond relative to propanol and subsequent urea-DTT extractions. This was not observed for Bonanza. This could indicate that, in certain varieties, there may be differential binding among the B hordein components. A similar portion of the total D hordein of each variety also was extracted in this solvent. This

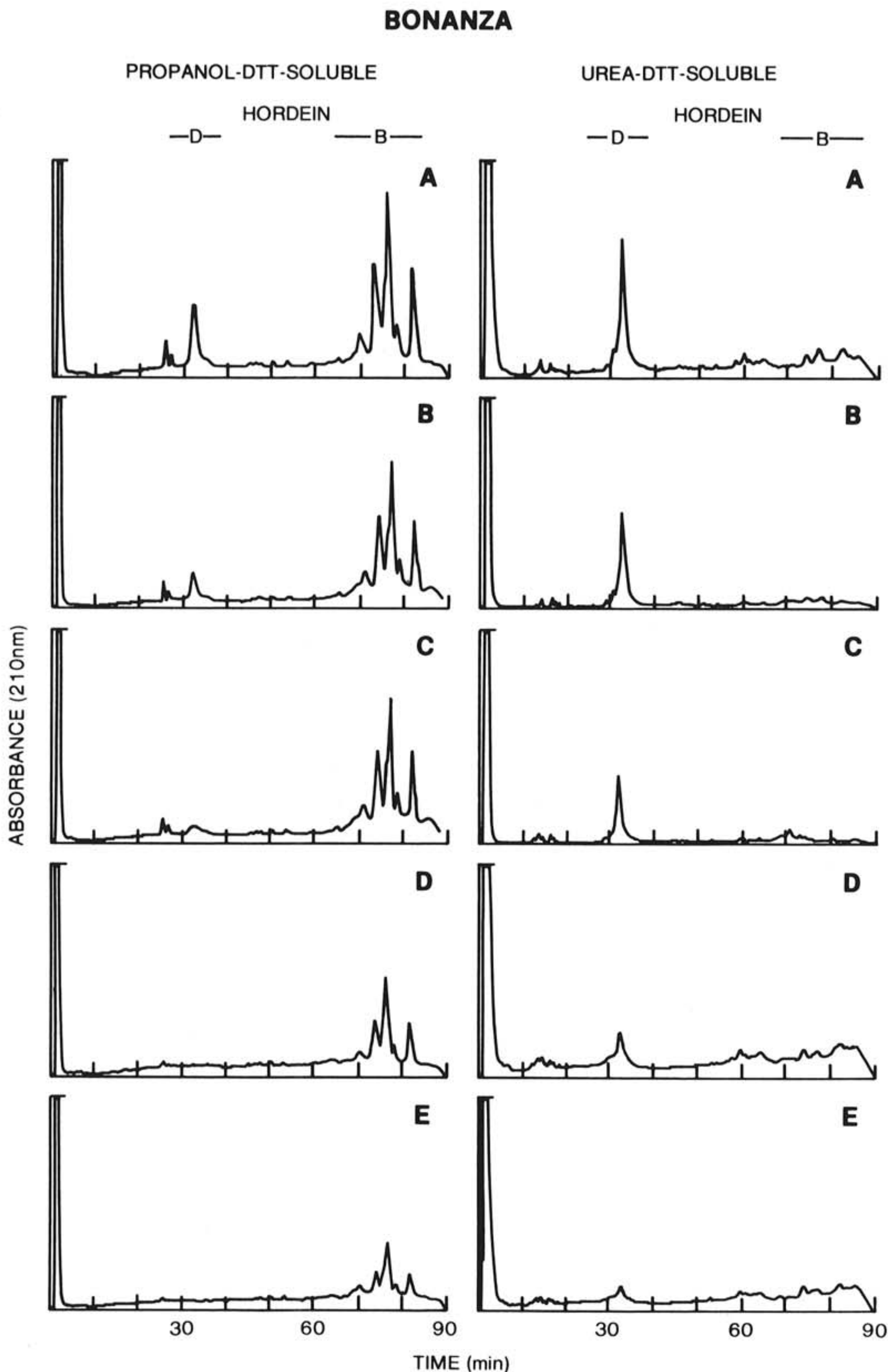


Fig. 6. Reversed-phase high-performance liquid chromatography separation of Bonanza barley and malt proteins extracted in the sequential extraction procedure using 50% 1-propanol + 1% dithiothreitol (DTT) (propanol-DTT-soluble) and 8*M* Urea + 1% DTT (urea-DTT-soluble). (**A**) sound grain, (**B**) two-day steep, (**C**) one-day germinated, (**D**) three-day germinated, and (**E**) five-day germinated.

proportion decreased rapidly during malting such that after two days of germination, it was zero in either variety.

As indicated by Baxter (1980, 1981) and more recently by Miflin et al (1983), the tendency of hordein to form aggregates may influence malting quality in a number of ways. Miflin et al (1983) found that the ratio of hordein extracted by propanol (hordein I) to propanol-DTT (hordein II) had some relationship to malting quality; i.e., the lower the ratio, the lower the quality. In this study, it was felt that it would be more appropriate and possibly more discriminatory to consider only the B hordein extracted by each solvent because this is the hordein component that would be involved primarily in disulfide bonding. As shown in Table V,

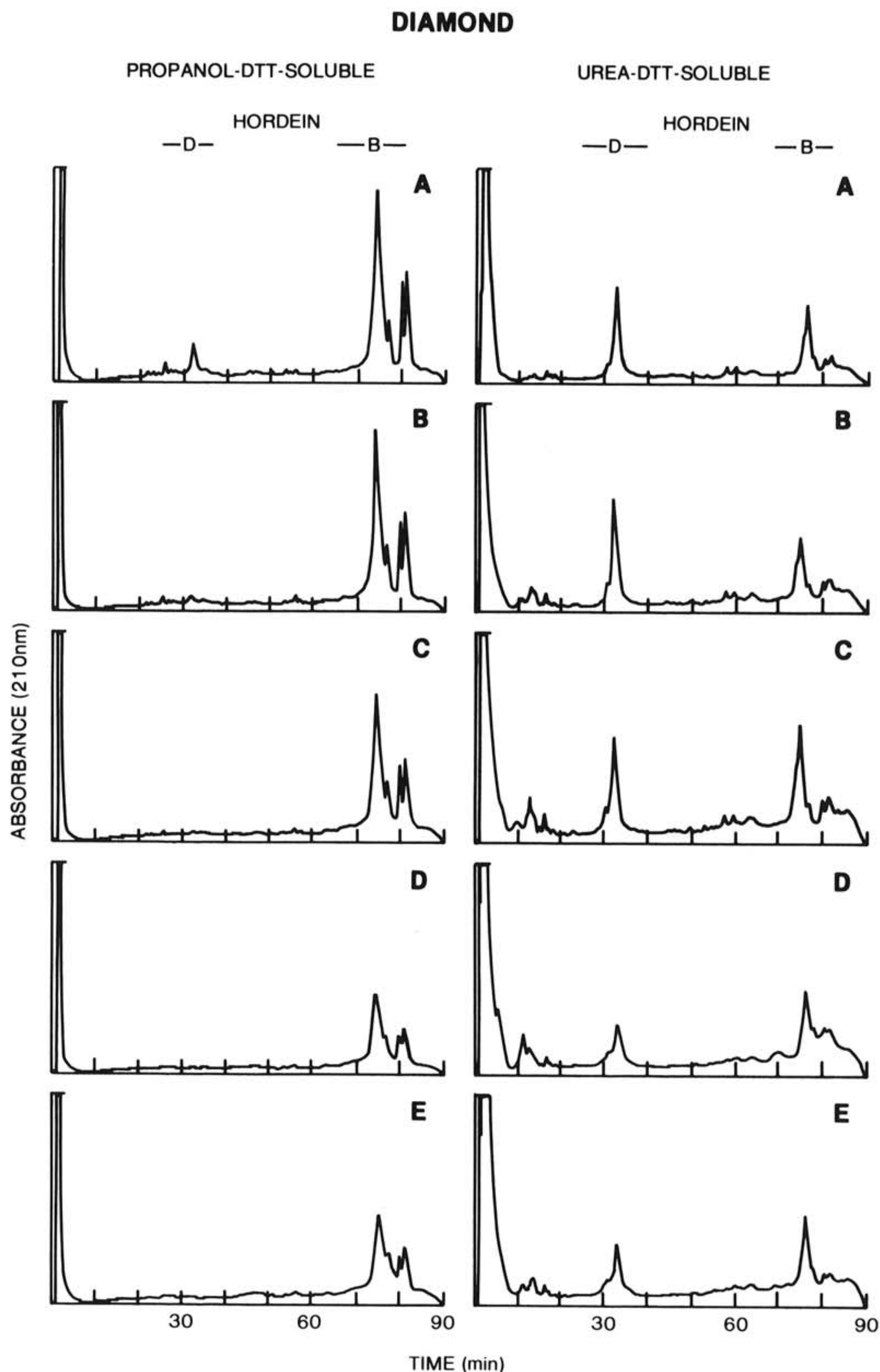


Fig. 7. Reversed-phase high-performance liquid chromatography separation of Diamond barley and malt proteins extracted in the sequential extraction procedure using 50% 1-propanol + 1% dithiothreitol (DTT) (propanol-DTT-soluble) and 8M urea + 1% DTT (urea-DTT-soluble). (**A**) sound grain, (**B**) two-day steep, (**C**) one-day germinated, (**D**) three-day germinated, and (**E**) five-day germinated.

Diamond exhibited a substantially lower ratio than Bonanza, which would appear to coincide with the relationship reported by Miflin et al (1983).

Final extraction with urea-DTT removed further hordein from both cultivars with a larger proportion being removed for Diamond as compared to Bonanza (Table IV). A major proportion (> 80%) of the total D hordein was extracted into this fraction. This proportion increased to 100% in both cultivars after two days of germination, and D hordein was detectable in this solvent throughout malting. In contrast, the D hordein extracted in the propanol-DTT was not detectable after two days germination. This suggests that a small portion of the D hordein is bound more loosely within the endosperm and becomes more available to proteolytic degradation. A small proportion of the total B hordein also was extracted but, as shown in Table IV, a substantially larger proportion was found for Diamond.

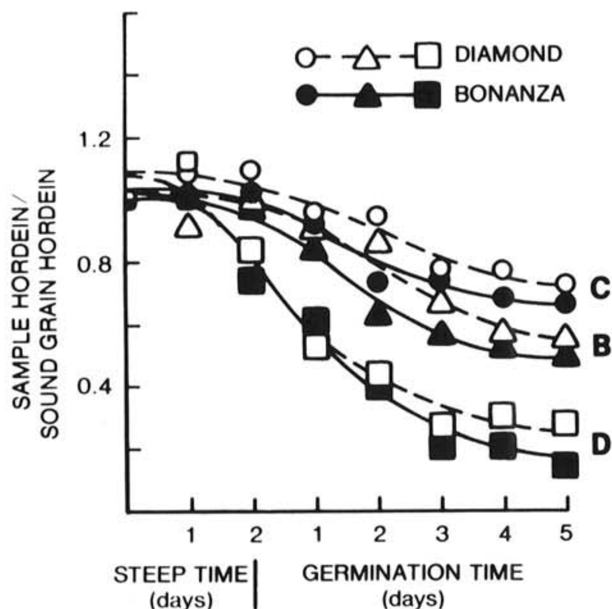


Fig. 8. Decrease in the B, C, and D hordeins during malting. Hordein composition was determined by reversed-phase high-performance liquid chromatography, and the decrease was relative to the initial composition in the sound grain.

As previously discussed, the relationship between the B hordeins extracted in different solvents may relate to malting quality. For this reason, B hordein extracted in urea-DTT also was compared to the B hordein extracted in propanol and propanol-DTT. As shown in Table V, these ratios again were significantly lower for the poor malting cultivar Diamond.

Recent studies by Smith and Simpson (1983) and Smith and Lister (1983) on SDS gel proteins have provided evidence linking D hordein to malting quality. RP-HPLC analysis of the SDS gel fraction prepared from Bonanza and Diamond sound grain (Fig. 9) indicated that the major protein components of the gels were D and B hordein. This is in agreement with the results reported by Smith and Lister (1983). Of interest also are the similarities between the hordein composition of the urea-DTT extracts (Figs. 6 and 7, A–E) and the SDS gel fraction (Fig. 9). Qualitatively both fractions had similar elution profiles. Quantitatively the D:B ratio was determined because the sulfur-containing hordeins may be involved in aggregation and thus related to quality. For both fractions, Bonanza had a higher ratio than Diamond (Table VI) in the sound grain and after malting. A decrease in this ratio during

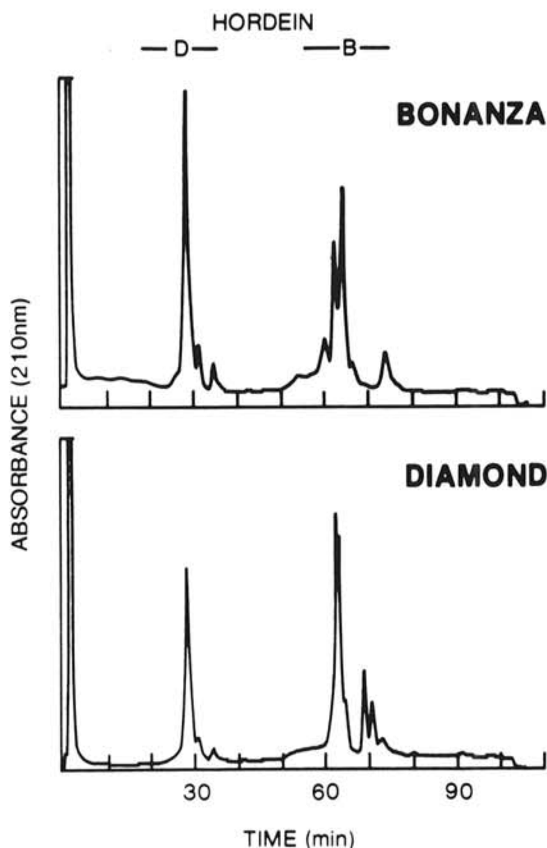


Fig. 9. Reversed-phase high-performance liquid chromatography separations of the sodium dodecyl sulfate gel proteins from Bonanza and Diamond sound grain.

TABLE VI
Ratio of D:B Hordein in Urea-Dithiolthreitol (DTT) Extracting Solvent and Sodium Dodecyl Sulfate (SDS) Gel Fraction

	D:B Ratio	
	Bonanza	**Diamond**
8*M* Urea + 1% DTT		
Sound grain	3.9:1	1.1:1
Germinated three days	1.3:1	0.4:1
SDS gel fraction		
Sound grain	0.9:1	0.5:1
Germinated five days	0.5:1	0.3:1

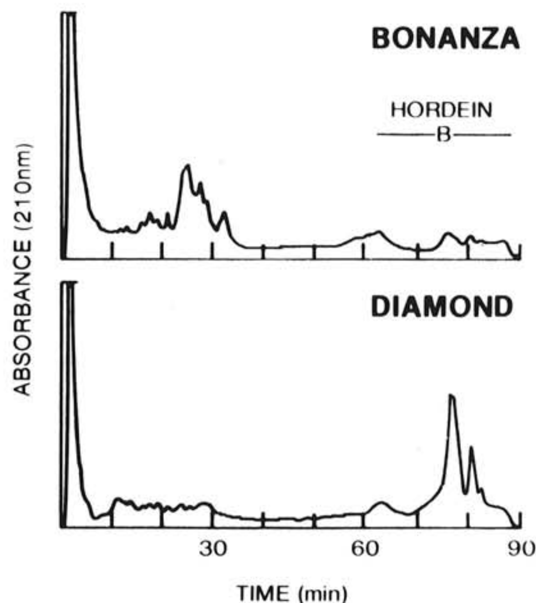


Fig. 10. Reversed-phase high-performance liquid chromatography separations of proteins present in a coarse-grind hot water extract prepared form the kilned malt of Bonanza and Diamond barley.

malting also was consistent for both fractions. A major difference, however, was that the SDS gel fraction contained significantly higher levels of B hordein than the urea-DTT extracts.

Because of the similarities between the extracts, it may be feasible to prepare the urea-DTT extract as an alternative to the SDS gel fraction. Preparation of the SDS gel fraction was very difficult for higher quality varieties and in particular for malted samples. In contrast, preparation of the urea-DTT extracts was relatively simple.

Analysis of the hot water extracts indicated that substantially more B hordein from Diamond was extracted and made its way into the wort (Fig. 10). Recent work has indicated that hordeins are important in chill haze formation (Asano et al 1983). Therefore, the presence of high levels of hordein in the hot water extract as determined by RP-HPLC also may prove useful as a quality determinant.

CONCLUSIONS

Malting and brewing quality consists of a group of different characteristics, and therefore a straightforward relationship with hordein protein characteristics would not be expected. SDSGPAGE and RP-HPLC analysis of hordein proteins have indicated that significant differences exist in the relative extractability characteristics among the B, C, and D hordeins within and between two barley cultivars of contrasting malting quality as shown in Tables IV, V, and VI. Further analysis of more barley cultivars with a wider range of quality will be necessary to determine if these characteristics represent a general relationship to malting as well as brewing quality.

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