Purification and Characterization of Barley D-Hordein[1]

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ABSTRACT

D-Hordein was purified from three barley varieties, and a glutelin of similar molecular weight was purified from one wheat variety by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis. These polypeptides were analyzed for amino acid composition and cleavage sites by sequence-specific proteases. None of the purified proteins fragmented under standard conditions of cyanogen bromide clevage, but they fragmented substantially when either D-hordein or glutelin was digested with trypsin, chymotrypsin, or *Staphylococcus aureus* V8 protease. Whereas amino acid analyses showed general similarity between the purified proteins, V8 protease showed a major difference in location of glutamate and aspartate residues between the D-hordeins and the purified glutelin.

The principle storage proteins of barley, the B-, C-, and D-hordeins, consist of alcohol-soluble proteins that are packaged into membrane-bound protein bodies late in endosperm development (Cameron-Mills 1980, Miflin et al 1981, Larkins and Hurkman 1978). Relatively nonpolar solvents were used to purify B- and C-hordeins by ion-exchange chromatography (Schmitt 1980), and by ion-exchange chromatography in chaotropic solutions followed by gel-exclusion chromatography (Shewry et al 1980). These techniques allowed gram-quantity purification of a few specific hordeins, but no general technique was discovered for the isolation of all the hordeins.

Recent technological advances allowed the use of microgram quantities of pure protein in amino acid analysis (Udenfried et al 1972), peptide mapping (Benson 1976, Cleveland et al 1977), and even microsequence analysis (Hunkapillar and Hood 1980). Preparative polyacrylamide gel electrophoresis techniques have been developed that allow small quantities of protein to be purified in one or two steps (Chrambach et al 1979, Hardman and Kane 1980, Allington et al 1978).

Shewry et al (1978) first used preparative electrophoresis to purify B- and C-hordeins. In the present investigation, a similar system was used to purify microgram quantities of D-hordein from the barley cultivars Bomi, Advance, and Hiproly and the high molecular weight (HMW) subunit with apparent molecular weight most similar to the barley D-hordeins, subunit 2, from the wheat cultivar Peak 72. Following purification, these polypeptides were subjected to amino acid analysis and peptide mapping.

MATERIALS AND METHODS

Chemicals

All chemicals used were of reagent grade, except acrylamide, bisacrylamide, and urea, which were sequenal grade. DPCC-trypsin and chymotrypsin were from Sigma, *Staphylococcus aureus* V8 protease was from Miles Laboratories, and cyanogen bromide was from Worthington.

Protein Extraction

Barley flour was prepared from whole seed ground in a Udy mill to pass a 0.5-mm screen. Twenty-gram samples of flour were sequentially extracted once with 60 ml of petroleum ether (bp 35–60°C) for 1 hr at 4°C, three times with 60 ml of 0.5 *M* NaCl at 4°C, and three times with 55% 2-propanol containing 0.37 *M* Tris-Cl, pH 8.8, and 4 m*M* dithiothreitol. The propanol extracts were bulked, and prolamins precipitated by the addition of two volumes of glass-distilled water followed by overnight storage at 4°C. Precipitated hordeins were recovered by centrifugation at 20,000 × *g* for 20 min.

Hordeins were solubilized by boiling in sample buffer containing 1% (v/v) SDS, 10% (v/v) glycerol, 0.0625 *M* Tris-Cl, pH 6.8, 4 m*M* dithiothreitol (Laemmli 1970) until dissolved to yield a protein concentration of approximately 10 mg/ml. Aliquots were centrifuged at 12,800 × *g* for 2 min before electrophoresis.

Preparative Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (1970) in 12.4% acrylamide, 0.1% bisacrylamide gels that had dimensions of 11 cm × 18 cm × 3 mm. A 2-cm stacking gel (3% monomer, 0.08% bisacrylamide) containing one large well overlaid the resolving gel. Samples were electrophoresed at 30 mA per gel until the tracking dye reached the gel bottom (approximately 5 hr).

Protein Visualization

Prolamins were made visible in preparative SDS-PAGE gels by immersing the gels in cold (4°C) 1 *M* KCl for 5 min. Prolamin bands appeared white on a nearly transparent background.

Protein Elution

Bands corresponding to D-hordein were cut from the preparative SDS-PAGE gels with a razor blade. The gel slice was placed in dialysis tubing containing 10 ml of 0.37 *M* Tris-Cl, pH 8.8, 4 m*M* DTT. Dialysis bags were embedded in 1% agarose (low M_r, Bio-Rad) containing the same buffer. Protein was eluted by employing a 3 V/cm potential over 8 hr. Completion of protein elution was confirmed by restaining the gel slice in 0.1% Coomassie Brilliant Blue R-250 in 20% methanol, 6% acetic acid overnight, and destaining in 25% methanol, 8% acetic acid.

Amino Acid Analysis

Duplicate 50-μg samples were hydrolyzed for 21 hr at 110°C under N_2 with 1 ml of 6*N* HCl containing 0.5% 2-mercaptoethanol (Keutman and Potts 1969). A Beckman model 121 MB analyzer was used to determine amino acids.

Peptide Preparation

Cyanogen bromide (CNBr) cleavage was performed following the general procedure of Gross and Witkop (1967). The protein (100 μg) was dissolved in 0.5 ml of 72% formic acid and treated with CNBr (20 mg/ml) at room temperature for 15 hr in the dark. The reaction mixture was diluted 10-fold with water and dried under vacuum. Acid hydrolysis was monitored by incubating parallel samples in 72% formic acid without CNBr. Bovine serum albumin was digested in parallel with the hordein samples as a positive control.

Enzymatic protein digestions were performed according to the method of Cleveland et al (1977). Approximately 10 μg of D-hordein or HMW glutelin was digested with varying amounts of *Staphylococcus aureus* V8 protease for 150 min, and time-course

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digests were performed using 10 μg of D-hordein and 0.25 μg of DPCC-trypsin or chymotrypsin for up to 5 hr, followed by increasing the enzyme concentration 10-fold and incubating for 16 hr.

Peptides were solubilized in sample buffer to yield an approximate concentration of 1 mg/ml. Peptides were separated in 12–24% linear polyacrylamide gradients; acrylamide/bisacrylamide ratios were used as before. Apparent molecular weights were determined by comparison with the mobility of phosphorylase (93,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (18,000),

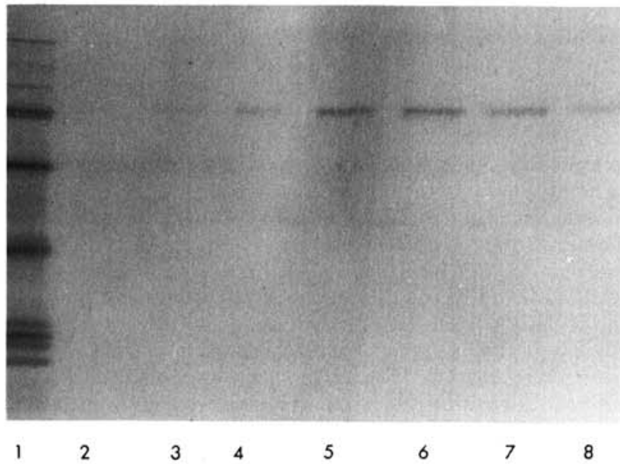

Fig. 1. Concentration range of Bomi D-hordein. Lane 1, molecular weight markers; lane 2, 5 μl of Bomi D-hordein extract; lane 3, 10 μl of Bomi D-hordein extract; lane 4, 20 μl of Bomi D-hordein extract; lane 5, 50 μl of Bomi D-hordein extract; lane 6, 100 μl of Bomi D-hordein extract; lane 7, 50 μl of Bomi D-hordein extract; lane 8, 25 μl of Bomi D-hordein extract.

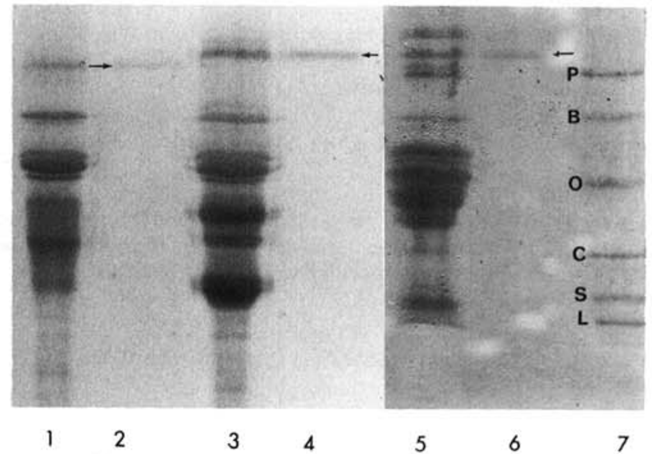

Fig. 2. Purified D-hordein fractions. Lane 1, Hiproly total hordein; lane 2, Hiproly purified D-hordein; lane 3, Bomi total hordein; lane 4, Bomi purified D-hordein; lane 5, peak 72 total alcohol-soluble proteins; lane 6, peak 72 high molecular weight glutelin no. 2; lane 7, molecular weight markers including phosphorylase (93,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (18,000), and lysozyme (14,400). Purified storage proteins are indicated by arrows, marker proteins are indicated by lowercase letters.

Fig. 3. Comparison of Coomassie blue R-250 staining of purified C-hordein with silver staining of purified D-hordein. Lanes 1–4, proteins stained with silver; lanes 5–8, proteins stained with Coomassie blue. Lanes 1 and 8, molecular weight markers, identity indicated by lowercase letters; lanes 2 and 7, Advance purified D-hordein; lanes 3 and 6, Bomi purified D-hordein; lanes 4 and 5, Hiproly purified D-hordein indicated by arrows.

and lysozyme (14,400) purchased from Bio-Rad. Figure 1 also includes B-galactosidase (116,250) and myosin (200,000). The apparent molecular weight of intact D-hordein was determined in 12.4% polyacrylamide gels, using the same standards.

Protein Staining

Polyacrylamide gels were stained overnight in 20% methanol and 6% acetic acid containing 0.1% Coomassie Brilliant Blue R-250 and were destained in 25% methanol, 8% acetic acid. The silver-staining system used was that of Wray et al (1981).

RESULTS AND DISCUSSION

Purification of D-Hordein

Preparative SDS-PAGE was used to purify microgram quantities of D-hordein from the barley cultivars Bomi and Hiproly (Fig. 2). The apparent molecular weights of these proteins were shown to be 92,800, and 85,000, respectively, and the molecular weight of the HMW glutelin purified from the wheat cultivar Peak 72 was similarly estimated to be approximately 98,000 (Fig. 2).

Various quantities of purified hordein from Bomi were used to estimate contamination (Fig. 1). When Coomassie blue was used, a sample application 10 times higher than that required for visibility showed no evidence of substantial contamination.

Previous authors regarded D-hordein as a nonprolamin contaminant, or as an aggregate of lower molecular weight prolamins linked through disulfide bonds (Cameron-Mills 1980). Undoubtedly, this was due to poor extractability in solvents lacking reducing agents, and to relatively low content in the seed (Field et al 1982).

A highly sensitive silver-staining system (Wray et al 1981) was attempted on one-dimensional separations of D-hordein to assess the homogeneity of the preparations (Fig. 3). This photosensitive straining process was shown to have 100 times the sensitivity of Coomassie blue R-250 in proteins within polyacrylamide gels (Switzer et al 1979, Merrill et al 1981, Wray et al 1981). Not only did the Bomi and Hiproly D-hordeins fail to stain, they appeared to inhibit background silver reduction in the polyacrylamide, providing clear bands on a brown background. This silver-stain system was performed in an ammoniacal environment, reportedly leading to deposition of silver on glutamate and aspartate residues. The lack of staining of the D-hordeins suggested extremely low glutamate and aspartate content, an observation supported by the basic pI of the protein (Blake et al 1982, Field et al 1982) and the limited fragmentation of D-hordein by *Staphylococcus aureus* V8 protease, a protease that cleaves at the C-terminal side of glutamic and aspartic residues.

D-Hordein was stable to reduction and alkylation with iodoacetamide (Blake et al 1982). This demonstrated that this protein was not structurally homologous to the HMW gliadin reported by Bietz and Wall (1980), which was labile to disulfide reduction, D-Hordein appeared to be a single polypeptide with apparent molecular weight of approximately 100,000. D-Hordein appeared to be similar to the HMW glutelins of wheat, specifically to the glutelins produced by genes on chromosome 1B as discussed by Holt et al (1981). Not all varieties examined produce a single D-hordein polypeptide per allele. In Bomi, two proteins slightly different in apparent molecular weight were encoded by the *Hor*-3 locus. A similar phenomenon was noted for the HMW glutelins encoded by chromosome 1B in wheat by Brown et al (1979).

TABLE I
Amino Acid Analyses of D-Hordein and High Molecular Weight Glutelin[a]

Amino Acid	Barley			Wheat
	Bomi	Hiproly	Advance	Peak 72
Aspartic acid	4.2	3.4	3.6	4.3
Threonine	5.2	4.6	4.2	3.5
Serine	9.6	8.2	6.9	13.1
Glutamic acid	20.7	26.8	27.3	25.6
Proline	9.9	13.8	14.0	6.5
Glycine	26.9	20.4	14.6	20.2
Alanine	4.4	3.7	3.9	5.8
Valine	2.4	2.0	2.4	2.7
Methionine	1.4	0.3	1.6	0.3
Isoleucine	1.5	2.1	4.6	1.7
Leucine	4.3	5.0	7.4	3.4
Tyrosine	2.6	3.1	2.4	3.7
Phenylalanine	2.3	3.0	2.9	1.3
Lysine	1.8	1.7	1.7	1.0
Histidine	2.4	2.3	2.6	1.8
Arginine	3.0	2.4	2.4	4.0

[a]Tryptophan and cysteine not determined. All values are averages of duplicate analyses, performed on aliquots dialyzed for 48 hr against several changes of glass-distilled water. Values calculated to represent moles of amino acid per 100 moles of total amino acids. Asx and Glx include acid and amide forms.

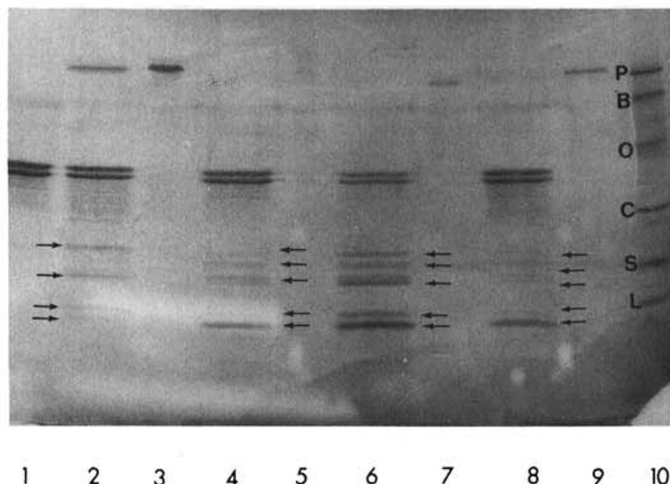

Fig. 4. *Staphylococcus aureus* V8 protease digestion of D-hordeins and higher molecular weight (HMW) glutelin. Lane 1, V8 protease incubated alone; lane 2, peak 72 HMW glutelin incubated with V8 protease; lane 3, Peak 72 HMW glutelin incubated alone; lane 4, Advance D-hordein incubated with V8 protease; lane 5, Advance D-hordein incubated alone; lane 6, Hiproly D-hordein incubated with V8 protease; lane 7, Hiproly D-hordein incubated alone; lane 8, Bomi D-hordein incubated with V8 protease; lane 9, Bomi D-hordein incubated alone; lane 10, molecular weight markers indicated by lowercase letters. Arrows indicate peptides produced by hordein or glutelin digestion by V8 protease.

Amino Acid Analysis

Amino acid analyses performed on these purified proteins showed broad similarity, but also differences that might be significant (Table I). Essential amino acid content was somewhat higher than in B- and C-hordeins, especially lysine (Shewry et al 1980, Schmitt 1980). The high glycine content may be characteristic of these proteins, as Khan and Bushuk (1979) have reported similar glycine contents for HMW glutelins, and Field et al (1982) have reported similar findings for D-hordein from the barley cultivar Sundance. The relatively low proline content of the purified Peak 72 HMW glutelin may merely reflect an unusual glutelin subunit, or it may deserve further study in analyzing the source of the high dough strength characteristic of this cultivar.

Peptide Mapping

Cyanogen bromide digestion of Bomi, Advance, and Hiproly D-hordein failed to fragment the proteins significantly, although an unknown number of small peptides may have been produced from the N- or C-termini. D-Hordein was fragmented into peptides too small to resolve after 16 hr of digestion by chymotrysin, while DPCC-trypsin left two peptides of apparent molecular weight greater than 15,000 from Bomi and only one from Hiproly (data not shown).

Staphylococcus aureus V8 protease treatment yielded five major peptides from all three barley D-hordeins tested and four from the glutelin subunit (Fig. 4). These peptides were separated solely by apparent molecular weight as determined by gradient SDS-PAGE.

Although the peptides shown in Fig. 4 represented the most

complete digestions performed in this study, some glutamic or aspartic acid residues may have remained undetected by the protease due to secondary configuration of the substrates. Also, each of the peptide bands observed on the gradient SDS-PAGE gel may represent more than one unique peptide. Recent investigations into the structure of zein, the maize storage proteins, demonstrated the presence of a conserved sequence corresponding to 20 amino acids which was repeated nine times in both of two completely sequenced zein genes (Marks and Larkins 1982). A similarly repeated internal sequence was suggested for wheat prolamins by Kasarda (1980). Internal repetition could provide evenly spaced rare amino acids, such as aspartic and glutamic acids in D-hordein. Upon cleavage with V8 protease, such even spacing would produce multiple peptides with identical molecular weights and, therefore, mobilities in SDS-PAGE.

Though both trypsin and chymotrypsin were expected to produce peptides too small to resolve on polyacrylamide gels, the lack of internal cleavage of D-hordein by cyanogen bromide was surprising. The methionine residues in the proteins either were located near the amino and carboxy termini of D-hordein or were unavailable for cleavage under the conditions reported in Materials and Methods. A similar finding was reported by Field et al (1982) upon attempted cysteine-dependent cleavage of D-hordein.

The molecular weights for the D-hordeins were somewhat lower than those reported by Field et al (1982) and slightly higher than those reported by Blake et al (1982). Those reported here range from 85,000 to 95,000, whereas those determined by Field et al (1982) were approximately 105,000 and those reported by Blake et al (1982) were 83,000 and 91,000. The variation between the estimates previously reported by this laboratory and the current estimates related directly to the addition of phosphorylase b as a molecular weight standard. The variation between our estimates and those provided by Field et al (1982) probably was related to differences in analytical techniques employed. Field et al (1982) have further reported analytical ultracentrifugation molecular weight estimations of approximately 54,000 for D-hordein. This disparity between results indicated that either SDS-PAGE or analytical ultracentrifugation or both were inadequate determinants for hordein molecular weights. SDS-PAGE was shown to produce artifactual apparent molecular weights for some proteins (Robards 1979). Nonetheless, the production of digestion products from *Staphylococcus aureus* V8 protease digestion whose apparent molecular weights sum to at least 70,000 suggested that the estimate of Field et al (1982) may also require further scrutiny.

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