

Determination of Molecular Weights of C Hordeins by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

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ABSTRACT

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The molecular weights of nine C hordein proteins, including all of the major components identified by SDS-PAGE have been determined by MALDI-MS. Most were in the range 43,561 to 46,857, with a single component of M_r 57,606. These are higher than the masses previously

reported based on the sequences of cloned genes. This indicates that reliance on masses determined solely by gene sequencing is not valid, presumably because these may not encode the major expressed proteins.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for the determination of protein molecular weights. However, it has been known for some time that cereal prolamins may migrate anomalously in this system, leading to overestimation for the molecular masses of prolamins of the Triticeae (barley, wheat, rye) and underestimation for the α -zeins of maize (Bunce et al 1985; Coleman and Larkins 1999). Although the molecular masses of many proteins are now routinely determined by analysis of the corresponding gene sequences, the complexity of cereal prolamins and the difficulty in assigning individual proteins to specific genes present in complex multigene families means that there remains a continued requirement to determine precise masses by other methods.

The S-poor prolamins comprise the C hordeins of barley, ω -secalins of rye and ω -gliadins of wheat. Each is a complex mixture of components and, for ω -secalins and ω -gliadins, it is possible to compare the masses of components determined by three methods: SDS-PAGE, MALDI-TOF MS analysis of purified proteins, and prediction from gene sequences. For ω -secalins the masses predicted from gene sequences (39,000) (Hull et al 1991; Clarke and Appels 1999) and determined by MALDI-TOF MS (39,117) (Rocher et al 1996) are similar and lower than those determined by SDS-PAGE (48,000 to 53,000) (Kasarda et al 1983). In contrast, the masses calculated for ω -gliadins encoded by the A and D genomes (\approx 30,400 and 39,200) (Hsia and Anderson 2001) are lower than those determined by either SDS-PAGE (44,000 to 78,000) (Kasarda et al 1983) or MALDI-TOF MS (41,900 to 42,800 for D genome-encoded ω -gliadins) (DuPont et al 2000; Seilmeier et al 2001). Masses of C hordeins have been determined by SDS-PAGE and gene sequencing, and here the discrepancy is even greater. Thus, SDS-PAGE showed components ranging in M_r from \approx 55,000 to 70,000 (Shewry et al 1980), while gene sequencing has given masses from 28,000 to 39,000 (Entwistle 1988; Entwistle et al 1991; Sayanova et al 1993). To resolve this conflict and to establish the validity of basing the masses of S-poor prolamins on gene sequences, we have determined the masses of a range of purified C hordein components by MALDI-TOF MS.

MATERIALS AND METHODS

Purification of C Hordeins

A preparation enriched in C hordeins was extracted from milled grain of the mutant barley line 'Risø 56' by stirring with 10 volumes 70% (v/v) ethanol for 1 hr followed by centrifugation at 5,000 \times g for 10 min. The extraction was performed twice, the supernatants combined and dialyzed against 1% (v/v) acetic acid and freeze-dried. The preparation (5 g) was dissolved in 10 mM glycine-acetate buffer, pH 4.6, containing 3M urea and applied to a column (50 \times 4.4 cm) of carboxymethyl cellulose (CMC) equilibrated in the same buffer. Elution was achieved using a linear gradient of 0–25 mM NaCl followed by 25 mM NaCl, all dissolved in the same buffer. Fractions were examined by lactate-PAGE, pooled, dialyzed against 1% (v/v) acetic acid and freeze-dried. The purified C hordein fraction was then separated into individual components by a further separation on CMC using 250 mg of protein, a 1 \times 30 cm column, and a 0–30 mM linear gradient of NaCl.

Final purification for mass spectrometry was achieved using a preparative C₅ reversed-phase HPLC column (10 \times 250 mm) with 5- μ m particle size and 30-nm pore diameter. Elution was with a linear gradient of 32.5–50% (v/v) acetonitrile mixed from two reservoirs containing water plus 0.07% (v/v) trifluoroacetic acid (TFA) and acetonitrile plus 0.05% (v/v) TFA. Peaks were freeze-dried and analyzed for purity by analytical HPLC using a C₅ column (4.6 \times 250 mm), and by SDS-PAGE and lactate-PAGE using the methods of Laemmli (1970) and Clements (1987) as described by Shewry et al (1995).

Mass Spectrometry

MALDI mass spectra were acquired on a Voyager Elite-DE time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a UV nitrogen laser (λ = 337 nm). The instrument was operated in linear mode. Sample preparation was made according to the dried droplet method (Kussman et al 1997), using 2,6-dihydroxyacetophenone (2,6-DHAP) as the matrix. Protein fractions were dissolved in 50% (v/v) acetonitrile, 0.1% (v/v) aqueous TFA to provide a final concentration of \approx 10 pmol/ μ L.

TABLE I
Molecular Masses of C Hordeins Determined by MALDI-MS

Protein ^a	M_r	Protein ^a	M_r
c	57,606	h	46,518 and 46,750
d	45,747	i	46,679 and 46,739
e	43,561 and 43,792	j	46,498 and 46,709
f	43,779 and 43,842	k	46,699
g	46,763 and 46,857		

^a Protein designations (c–k) correspond to Fig. 1.

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The matrix solution was prepared by dissolving 2,6-DHAP in 50% (v/v) acetonitrile, 0.1% (v/v) aqueous TFA at a concentration of 50 $\mu\text{g}/\mu\text{L}$. Spectra were obtained in positive mode at an acceleration voltage of 25 kV and a delay time of 600–800 nsec. Spectra from about 150 laser shots were averaged to improve the signal-to-noise level. Mass assignment was made using creatine phosphokinase chain B from rabbit brain (M_r 42,659) (Pickering et al 1985) as external standard.

RESULTS AND DISCUSSION

C hordein comprises $\approx 70\%$ of the total hordein fraction in the barley mutant Risø 56 (Kreis et al 1983), which facilitates the purification of individual components. SDS-PAGE shows a major group of bands of $M_r \approx 43,000$ to 48,000 and a minor band of $M_r \approx 57,000$ (Fig. 1B, track b), but 2-D isoelectric focusing/SDS-PAGE shows the presence of at least 20 individual components (see separation reported for cv. Sundance, which has the same C hordein allele as Risø 56 [Shewry et al 1985]).

In the present study, nine C hordein fractions (c–k) were prepared. Lactate-PAGE (Fig. 1A) and SDS-PAGE (Fig. 1B) showed that most contained single major components, but at least two major components were present in fraction d and additional minor components in some other fractions. MALDI-TOF MS gave single masses

for fractions c, d, and k, whereas the other fractions contained at least two components (Table I). Fraction c corresponded to the lower mobility band separated by SDS-PAGE and gave a mass of 57,606. In contrast, the other fractions all corresponded to the major group of bands separated by SDS-PAGE and these gave lower masses ranging from 43,561 to 46,857. MALDI-TOF MS has an intrinsic uncertainty for proteins of this mass of $\approx 0.1\%$; hence, these values should be regarded as estimates.

The M_r values determined for C hordein by MALDI-TOF MS are lower than those from SDS-PAGE (55,000–70,000) (Shewry et al 1980) but higher than those reported based on gene sequences (28,000–39,000), which suggests that genes encoding typical C hordein proteins so far have not been isolated. Indeed, the gene isolated by Entwistle (1988) appears to be a pseudogene as it contains an 'in frame' stop codon. A similar discrepancy exists between the masses predicted for ω -gliadins encoded by genomic clones originating from chromosome 1A or 1D (30,400–39,200) and masses determined by MALDI-TOF MS for chromosome 1D-encoded ω -gliadins (41,900 to 42,800) (Hsia and Anderson 2001; Seilmeier et al 2001). In contrast, the masses determined for ω -secalins using these two approaches are similar, $\approx 39,000$ (Hull et al 1991; Rocher et al 1996; Clarke and Appels 1999).

We conclude, therefore, that MALDI-TOF MS is the preferred method for determination of the masses of S-poor prolamins, as SDS-PAGE and prediction from gene sequences may give anomalously high and low values, respectively.

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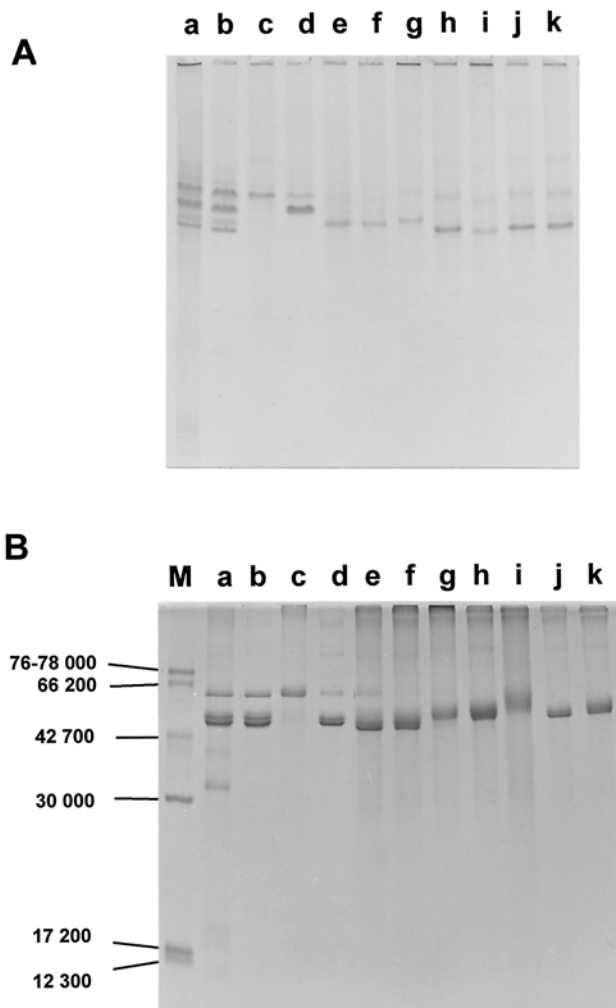


Fig. 1. Lactate-PAGE (A) and SDS-PAGE (B) patterns of C hordein fractions: (a) 70% ethanol extract of Risø 56; (b) C hordein proteins after ion-exchange chromatography; (c–k) individual C hordein fractions purified by HPLC; (M) molecular weight markers. Ovotransferrin (M_r 76–78,000), albumin (M_r 66,200), ovalbumin (M_r 42,700), carbonic anhydrase (M_r 30,000), myoglobin (M_r 17,200), and cytochrome c (M_r 12,300).

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