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Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry Analysis of Zeins in Mature Maize Kernels

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A high-throughput method has been developed to allow rapid analysis of maize seed storage proteins by matrix-assisted laser desorption time-of-flight mass spectrometry. The extraction solution containing an organic solvent, a reducing agent, and a volatile base has been optimized to enable extraction of all classes of zein proteins (α-, β-, γ-, and δ-). A near-saturating concentration of matrix, 2-(4-hydroxyphenylazo)benzoic acid, was necessary to obtain strong peaks for the most lipophilic zeins, the α-zeins. Zein proteins with small mass differences, difficult to separate by sodium dodecyl sulfate polyacrylamide gel electrophoresis, were resolved through this analysis. Mass signals corresponding to the 10-kDa δ-, 15-kDa β-, 16-kDa γ-, 27-kDa γ-, and several 19 and 22-kDa α-zeins were detected. The zein identities were further confirmed by the association of the number of cysteine residues in each zein MS peak, as determined by iodoacetamide derivatization, with the number predicted from its coding sequence. The relative zein abundance in the zein MS peaks was also correlated with the relative zein EST abundance among endosperm EST libraries. This method was utilized to examine the zein composition of a number of corn inbred lines and opaque mutants.

KEYWORDS: MALDI-TOF MS; zeins; prolamins; maize kernels

INTRODUCTION

Prolamins are the major storage proteins of all cereal grains except rice and oats (*1*) and are named as such due to their high proline and glutamine content. These proteins are characteristically localized in the endosperm of the seed. Their solubility in organic solvents, such as alcohols, facilitated their initial isolation and characterization (*2*). Additional prolamins, which are not alcohol soluble in their native state due to extensive cross-linking by cysteine disulfide bonds, were later found to be alcohol-soluble if extracted at a higher pH and under reducing conditions (*3*). In contrast, the insolubility of prolamins in water or aqueous salt solutions is a biologically and physically significant characteristic for their successful storage and an important consideration in their proper definition (*4*). In maize, prolamins, also known as zeins, comprise nearly 60% of the total seed proteins and are subdivided into distinct categories, α-, β-, γ-, and δ-zeins, based on their solubility and related protein structure from deduced nucleotide sequences (*5*, *6*). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), sometimes accompanied with immunodetection, is the most common method of analyzing zeins (*7*, *8*). For zeins with nearly identical molecular weights, varying merely in charge, or solubility, techniques such as isoelectric focusing (*9*) or HPLC (*10*) are also used.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful tool for resolving crude mixtures of proteins without using chromatographic separation. Proteins in the low picomole range can be detected within a few minutes, yielding results that are easy to interpret. Initial MALDI-TOF MS of crude alcohol prolamin extracts was developed to examine the gluten prolamins from wheat (gliadins), barley (hordeins), rye (secalins), and oats (avenins) because of their implication in celiac disease (*11*−*17*). MALDI-TOF MS with sensitivity equivalent to or exceeding that of ELISA procedures has been achieved for gliadins and avenins (*14*, *15*). Other important applications of MALDI-TOF MS to cereal prolamins include the rapid detection of prolamin fractions with important food-processing properties, directed breeding for the desirable prolamin fractions, and cereal variety identification (*18*−*20*).

The application of MALDI-TOF MS to crude prolamin extracts from corn is of interest because the resolution of zeins by conventional separation methods such as SDS-PAGE is limited by the relatively narrow zein molecular weight distribution. Zeins in the inbred B73 ranges only from 14431 to 27128 Da, with the exception of a low-abundance 50-kDa γ-zein at 32882 Da (*8*). The α-zeins, in particular, which usually appear as two broad bands on SDS-PAGE gels at approximately 19 and 22 kDa, are each composed of a complex mixture of

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polypeptides encoded by multigene families (*7*). Mass differences by as few as a single amino acid in such a multigene family are easily distinguished by MALDI-TOF MS.

Herein, we report a novel method of analysis and identification of zeins from crude maize kernel prolamin extracts by MALDI-TOF MS. We have used a maize inbred line, B73, for primary studies and found that the identity and relative abundance of each zein were comparable to those of a previous study that analyzed cDNA libraries from developing B73 endosperm (*8*). In addition, we present preliminary zein MALDI-TOF MS results from seven inbred varieties and two opaque mutants.

MATERIALS AND METHODS

Zein Extraction. Fifty milligrams of ground kernel powder was pre-extracted with two 0.5-mL washes of hexane to remove oil. After the hexane had evaporated, the samples were stored overnight at −20 °C. Zein proteins were extracted from the corn meal with 2.5 mL of extraction solution [60% acetonitrile (ACN), 25 mM ammonium hydroxide, and 10 mM dithiothreitol (DTT)], at 60 °C for 1 h with shaking every 15 min. The DTT was added immediately prior to use of the extraction solution. After incubation, the homogenates were centrifuged at 2000*g* for 5 min and the supernatants removed for analysis.

For larger numbers of samples, 24-mg samples of ground kernel powder were weighed into 96-well deep well plates with 2-mL well volumes (Whatman Polyfiltronics, Clifton, NJ). Next, 1.2 mL of extraction solution was added, and the plates were heat-sealed with aluminum foil (ABgene, distributed by Marsh, Rochester, NY). To prevent possible leakage between wells, the top of each plate was covered with a silicone foam pad compressed against the top of the microplate. During extraction at 60 °C the plates were shaken every 10 min. After incubation, the microplates were centrifuged for 5 min at 600*g* and the supernatants removed for analysis.

Iodoacetamide (IAA) Derivatization. Ten microliters of the zein extract was centrifuged briefly in a Speed Vac (Savant, Holbrook, NY) to remove most of the ammonia. The samples were incubated with 1 μL of 500 mM IAA for 10 min in darkness. This represented a 5-fold molar excess of IAA over DTT. After the incubation, the samples were diluted 10-fold with a 2-(4-hydroxyphenylazo)benzoic acid (HABA, Pierce, Rockford, IL) matrix (see next section), and 0.6 uL was applied to the MALDI target plate.

MALDI Sample Preparation. The matrix, HABA, was dissolved in 65% ACN and 0.3% trifluoroacetic acid (TFA). In cases when an internal standard was required, a 1/200th volume of 200 μM bovine erythrocyte carbonic anhydrase (CA, Sigma, St. Louis, MO) dissolved in 0.3% TFA was added to the matrix solution to give a CA concentration of 1 μM prior to addition of sample. HABA concentrations of 10, 20, 30, and 40 mM (2.42, 4.84, 7.27, and 9.69 mg/mL, respectively) in the matrix mix were tested. Unless indicated otherwise, all spectra shown were obtained using 40 mM HABA in the matrix mix.

The zein protein extracts were diluted 10-fold with matrix solution giving an equivalent of 2 mg of kernel powder/mL. A 0.6-μL aliquot of sample/matrix mixture was applied to a 384-well Voyager sample plate with a hydrophobic surface (Applied Biosystems, Framingham, MA) and dried for 10 min before the plate was put inside the mass spectrometer. For larger numbers of samples, the Applied Biosystems SymBiot I was programmed to remove 5-μL samples from a 96-well deep well microplate, mix the samples with 45 μL of matrix mix in a 384-well microplate, and apply 0.6 μL to the 384-well target plate.

MALDI-TOF MS Conditions. MALDI-TOF MS analyses were performed using an Applied Biosystems Voyager-DE PRO Biospectrometry Workstation with version 5.1 software. The mass spectrometer was operated using the following conditions: a 337-nm nitrogen laser, operating in the linear mode with a delayed extraction of 425 ns, a 25-kV accelerating voltage, and an ion flight path of 1.3 m. Spectra were averaged over 250 laser shots using 2 laser shots per position and 125 positions with a center bias search pattern. The laser intensity was set at 200 units above the threshold laser intensity.

Data Analysis. Spectra were analyzed with the Applied Biosystems version 4.0 Data Explorer software, according to the manufacturer's specifications. Prior to calibration, peaks were smoothed with a default method using a mass peak resolution of 600 and baseline correction. To determine the areas beneath each peak, zein peaks from printed copies of the spectra were cut out, weighed, and averaged.

Seed Sources. B37, B73, M14, Mo17, Oh43, W22, and W64A were obtained from the North Central Regional Plant Introduction Station, USDA-ARS, Ames, IA. Separate accessions of W64A and *opaque-2* and *mucronate* mutants in W64A background were provided by Dr. Brian Larkins, University of Arizona (Tuscon, AZ).

RESULTS AND DISCUSSION

Zein Extraction Conditions. *Organic Solvent Concentration.* The selective extraction of prolamins with organic solvent and the simultaneous exclusion of non-prolamin proteins greatly enhance the utility of the MALDI-TOF MS analysis. Pre-extraction of the kernel powder with water to remove albumins, and with aqueous salt to remove globulins, did not appear to be necessary to obtain zein spectra free of albumins and globulins. The choice of organic solvent concentration for zein extraction in this work was based on the studies of Bean et al. (*21*). These authors found that only relatively minor amounts of α-zeins were extracted with ACN concentrations lower than 50% (v/v). Optimal concentrations of ACN for α-zein extraction (under reducing conditions) were in the 60−70% range. Bean et al. also found that extraction of β-, γ-, and δ-zeins was high at 40−60% ACN. This is in agreement with the lower hydrophobicity of β-, γ-, and δ-zeins (*10*). Thus, alteration of the ACN concentrations can be used to selectively extract certain classes of zeins for more stringent analysis while leaving other classes largely insoluble. For example, the selective extraction of the β-, γ-, and δ-zeins could be obtained at ACN concentrations below 50%, whereas the selective extraction of α-zeins may also be possible at ACN concentrations above 60%. To include all zein classes for this analysis, 60% ACN was used in the extraction procedure.

Reducing Agent. Mature zein polypeptides from the inbred line B73 can have as few as a single cysteine residue or as many as 15 (**Table 1**). Particularly for zeins with the highest number of cysteine residues, efficient extraction is dependent on the reduction of disulfide bonds. The presence of 10 mM DTT in the sample extraction solution was sufficient to extract the 27-kDa γ-zein; when DTT was omitted from the extraction solution, this zein could not be detected in the spectra.

High pH. The 27-kDa γ-zein requires a high pH for efficient extraction. This higher pH is often achieved with 0.5% sodium acetate buffer, pH 10 (*3*), or 12.5 mM sodium borate buffer, pH 10 (*22*). However, in MALDI-TOF MS, sodium cations, which are not volatile, tend to form adducts with proteins, resulting in higher molecular weights and broader peaks. In our work, ammonia was chosen to raise the pH because ammonium adducts are likely to decay into ammonia gas and the molecular ion. The free ammonia is not normally detected in spectra. Likewise, ammonium carbonate and bicarbonate, and other more volatile organic amines at concentrations below 50 mM, do not usually cause adduct formation.

The measured pH of the 25 mM ammonium hydroxide solution used in our procedures is ∼10.85, but the actual pH during extraction is lower due to the buffering action of extracted proteins. Because the volatility of ammonia is increased at the higher temperatures used for zein extraction, the headspace of the extraction vessel was typically minimized to avoid pH

Table 1. Comparison of Molecular Weights and Number of Cysteine Residues in B73 Mature Zein Proteins Based on GenBank Accessions and Measurement with MALDI-TOF MS

name (abbreviation)[a]	GenBank accession no.	mol wt (calcd)	no. of cysteines in mature protein	mol wt of underivatized zeins observed in MS	mol wt of derivatized zeins observed in MS	mass increase after derivatization	units[c] of cysteines observed
10-kDa δ-zein (dz10)	AF371266	14431	5	14432	14715	283	4.96
15-kDa β-zein (bz15)	AF371264	17458	7	17125	17492	367	6.43
16-kDa γ-zein (gz16)	AF371262	17663	12	17714	18393	679	11.90
18-kDa δ-zein (dz18)[b]	AF371265	21220	3				
27-kDa γ-zein (gz27)	AF371261	21822	15	21793	22581	788	13.80
19-kDa α-zein B1 (az19B1)	AF371269	23359	2	23318	23428	110	1.93
19-kDa α-zein B3 (az19B3)	AF371271	24087	2	24069	24180	111	1.94
19-kDa α-zein D2 (az19D2)	AF371268	24706	1	24515	24564	49	0.86
19-kDa α-zein D1 (az19D1)	AF371267	24818	1	24644	24699	55	0.96
22-kDa α-zein Z1 (az22z1)	AF371274	26359	1	26308	26365	57	1.00
22-kDa α-zein Z5 (az22z5)[b]	AF371277	26701	1				
22-kDa α-zein Z3 (az22z3)	AF371275	26751	1	26741	26794	53	0.93
22-kDa α-zein Z4 (az22z4)[b]	AF371276	26923	1				
19-kDa α-zein B2 (az19B2)[b]	AF371270	27128	2				

[a] Based on all of the different zein ESTs detected in endosperm cDNA libraries (*8*), except 27-kDa γ-zein (gz50), 19-kDa α-zein B4 (az19B4), and 19-kDa α-zein B5 (az19B5). 27-kDa γ-zein (gz50) is located outside the mass range shown in the MS, whereas 19-kDa α-zein B4 (az19B4) and 19-kDa α-zein B5 (az19B5) produce truncated cDNA and in-frame stop codon, respectively. [b] Not identified in MS. [c] A 57.1 increase in molecular weight for each derivatized cysteine.

decline due to ammonia volatilization. The concentrations of ammonium hydroxide tested for zein extraction and subsequent MALDI-TOF MS of the zeins were 0, 25, 50, and 100 mM. When extractions were performed without ammonia, the 27-kDa γ-zein was not detectable in the spectra. The 25 mM ammonia concentration resulted in consistent γ-zein extraction. The 50 and 100 mM ammonia concentrations did not appear to result in further improvement of the spectra (data not shown). The use of nonvolatile anions, such as borate, phosphate, and SDS, known to suppress ionization, were avoided in the extraction solution.

Matrix Conditions. *Sufficient Matrix Acidity.* A pH of $\leq$2.0 in the final sample/matrix mix is required for efficient ionization of proteins during MALDI-TOF MS. To this end, 0.3% TFA, which has a pH of ~1.5, was added to the matrix mixture. Dilution of 1 volume of zein extract with 9 volumes of the 0.3% TFA (v/v) matrix mixture results in final concentrations of 2.5 mM ammonia and 36 mM TFA. This results in a mixture with at least a 14.4-fold excess of TFA to achieve a pH of $\leq$2.0.

Calibration Standard. The supplement of a known protein of known molecular weight in the matrix mix can serve not only for mass calibration but also as a semiquantitative reference peak for MALDI-TOF MS spectra. Most zein proteins have masses between 14431 (10-kDa δ-zein) and 26923 (22-kDa α-zein Z4), with the exception of the 32882 50-kDa γ-zein (*8*). An ideal internal molecular weight standard would have $[M + H]^+$ and $[M + 2H]^{+2}$ equally above and below the 22-kDa α-zein Z4 and 10-kDa δ-zein, respectively. Bovine erythrocyte carbonic anhydrase (CA) has a 29024 Da CA ($[CA + H]^+$) ion larger than the 22-kDa α-zein Z4; however, the 14512.5 Da CA ($[CA + 2H]^{2+}$) ion is so close to the 10-kDa δ-zein that complete resolution between the two proteins is not possible. Thus, in cases when the identification of 10-kDa δ-zein is necessary, CA is not useful for internal calibration. A low-level contaminant at ~15615 Da also appears to be present in the CA (**Figure 1**).

Martix Concentration. For a protein to be detected by MALDI-TOF MS, it must coprecipitate with matrix so that it can undergo desorption with the matrix after excitation with the laser. As the ACN organic solvent evaporates during sample preparation for MALDI-TOF MS, the zeins precipitate in a specific order. The 19- and 22-kDa α-zeins, requiring the highest concentration of organic solvent for solubility, precipitate first. The 15-kDa β-, 16-kDa γ-, and 10-kDa δ-zeins, requiring a lower concentration of organic solvent, precipitate next. Finally, the 27-kDa γ-zein, which is water-soluble following solubilization at high pH and reducing conditions, precipitates last.

Unlike the zeins, which precipitate differentially at a relatively narrower range of organic solvent concentrations, HABA precipitates gradually after it has reached saturation at a particular ACN concentration. With a starting ACN concentration high enough for all zein classes to be soluble (~60% ACN) and HABA present at near saturation (~40 mM HABA), HABA will begin coprecipitating with the α-zeins and continue coprecipitating with the β-, γ-, and δ-zeins as the ACN evaporates. In this instance, all four classes of zeins will appear in the spectra. However, at a lower HABA concentration, HABA will not begin to precipitate initially as the ACN evaporates until the most lipophilic zeins, the α-zeins, have precipitated. When the ACN evaporates further, HABA will reach saturation and coprecipitate with the less lipophilic β-, γ-, and δ-zeins, thus including them in the matrix. Therefore, the β-, γ-, and δ-zeins will appear in the spectra, but not the α-zeins. Finally, at very low initial HABA concentrations, only the least lipophilic zein, such as the 27-kDa γ-zein, will coprecipitate with the matrix. Thus, the initial HABA concentration strongly affects which classes of zeins appear in the mass spectrum. This also allows certain classes of zeins in the spectra to be preferentially displayed when a better resolution is required for these zeins.

Figure 1 shows the effect of four HABA concentrations on the relative prominence of zein peaks present in spectra of the maize inbred B73. The identification of each zein peak will be discussed in the next section. The CA standard peaks, $[CA + H]^+$ and $[CA + 2H]^{2+}$, are at the far right and far left of each spectrum, respectively. For normalization, the $[CA + H]^+$ peak has been adjusted to the same size for all four spectra. At 40 mM HABA, the 19- and 22-kDa α-zein peaks are prominent, but they decrease as the HABA concentration diminishes to 10 mM. Thus, the HABA concentration is an important variable to determine the relative abundance of zeins by MALDI TOF MS. As discussed in the next section, the relative abundance of each zein protein as determined by MALDI-TOF MS at the 40 mM HABA is correlated with the zein expression data obtained by Woo et al. (*8*).

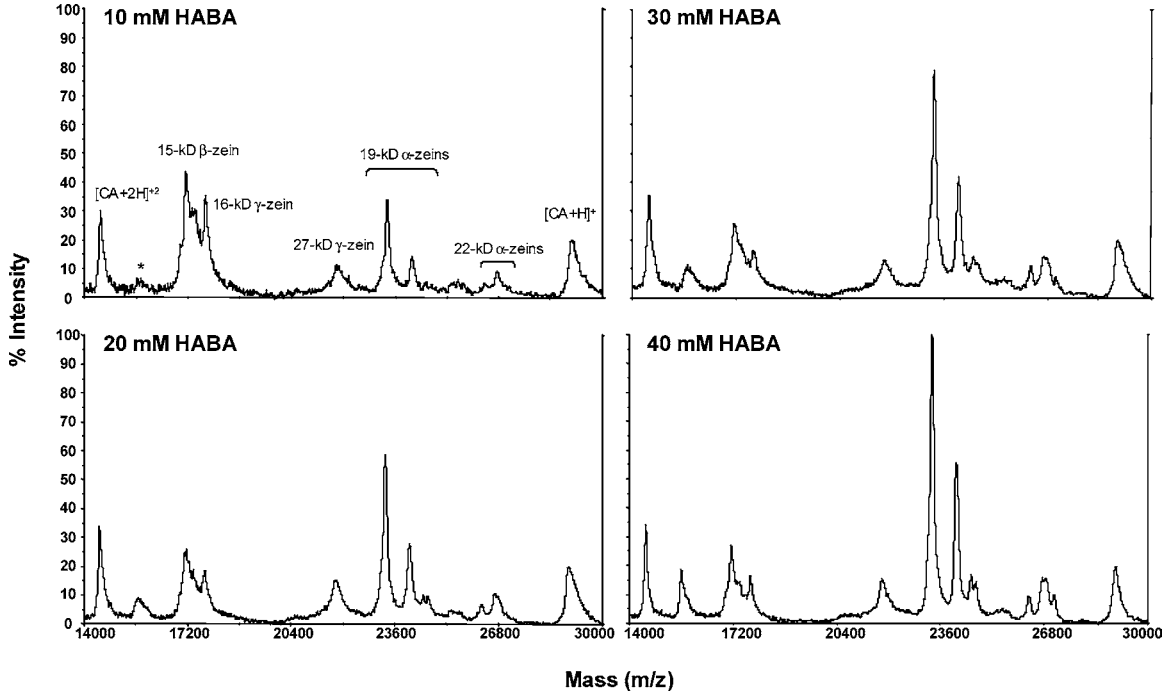

Figure 1. Effect of HABA matrix concentrations on detection of B73 zein mass spectrum. Each spectrum is labeled with the concentration of HABA matrix utilized. The identification of each zein peak is described in **Figure 2** and **Table 1**. The CA standard peaks, $[CA + H]^+$ and $[CA + 2H]^{2+}$, are at the far right and far left of each spectrum, respectively. For normalization, the $[CA + H]^+$ peaks were adjusted to the same size for all four spectra. The 15615 Da peak indicated by "∗" appears to be a contaminant from CA.

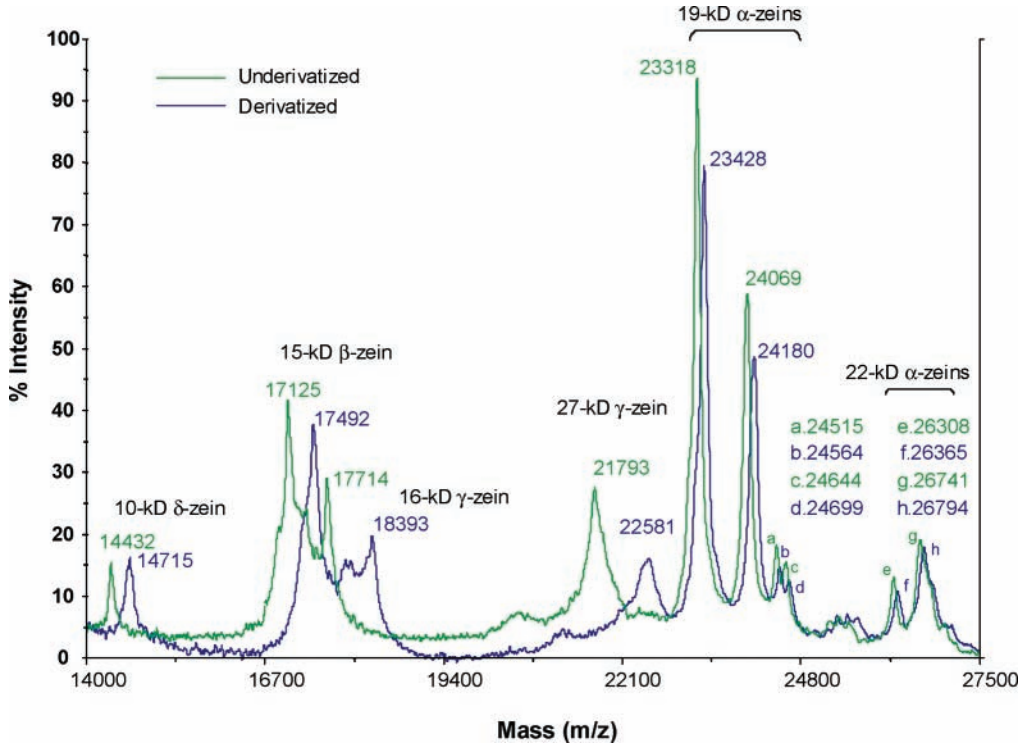

Figure 2. Two superimposed spectra (green, underivatized; blue, derivatized) showing the differential shifts of zein peaks following IAA derivatization. The peaks showing the largest shift to a higher mass have the largest number of cysteines. The CA standard was present in these samples due to the molecular weight proximity of $[CA + 2H]^{2+}$ and 10-kDa δ-zein. The HABA matrix concentration used was 20 mM.

Identification of Zein Peaks in the B73 Mass Spectra. A typical MALDI-TOF MS spectrum of B73 zein proteins (along with a separate run of IAA-derivatized zeins) is shown in **Figure 2**. The tentative identification of the zein peaks was initially based on a comparison of the molecular weight similarity of B73 zeins calculated from GenBank accessions (with the signal peptide removed) (*8*) and the MALDI-TOF MS observed zein masses (columns 3 and 5, **Table 1**). On average, the masses determined by the MALDI-TOF MS differed only 0.43% from the masses predicted from the deduced amino acid sequences. Zein identities were further verified by comparing the number of cysteine residues present in each zein, as determined by the IAA derivatization procedure, with the number of cysteine residues predicted from GenBank B73 zein coding sequences.

The number of cysteine residues was determined by IAA derivatization, which results in a mass increase of 57.1 Da for each cysteine residue. **Figure 2** shows two superimposed spectra from B73 of underivatized (green) and derivatized (blue) samples. The upward mass shift associated with the derivatized sample was used to calculate the number of cysteine residues in each zein peak. In **Table 1**, the masses of underivatized and derivatized B73 zein peaks from **Figure 2** are shown in columns 5 and 6. The resulting mass increase is shown in column 7 and the calculated number of cysteine residues in column 8. Overall, the number of cysteine residues determined by MS compares favorably to the number predicted by the deduced amino acid sequence.

Several low-abundance zeins were not identified, including 18-kDa δ-zein, 22-kDa α-zein Z5, 22-kDa α-zein Z4, and 19-kDa α-zein B2 (**Table 1**). The 22-kDa α-zeins with similar molecular weights and an identical number of cysteine residues are difficult to distinguish on the present spectra. It is likely that additional peaks could be identified by improved resolution in the 22-kDa α-zein region. A small peak is observed near the predicted 18-kDa δ-zein (*m*/*z* at 21220) in both underivatized and derivatized spectra, but it appears to be too low in molecular weight and does not appear to have the correct number of cysteine residues to identify this peak as the 18-kDa δ-zein.

Relative Abundance of Zeins in B73. Ideally, MALDI-TOF MS provides both qualitative and quantitative information for protein analysis. We determined the relative abundance of zeins in the B73 spectra by measuring their peak areas from the MS spectra and comparing their abundance to the percent of cDNAs sequenced from endosperm cDNA libraries (expressed sequence tags, ESTs), which were determined to encode zein proteins obtained by Woo et al. (*8*). The zein molecular weights were plotted against their calculated peak areas as well at their relative abundance among the endosperm cDNAs (% EST). These plots are depicted in **Figure 3**. The parentheses in **Figure 3** are the relative peak areas or % EST abundance calculated for each zein. Other than the 18-kDa δ-zein and 22-kDa α-zeins, the identities of which are still uncertain in the spectra, zeins with higher expression levels, in general, correlate with larger peak areas in the MALDI-TOF MS analysis. The only exception is 27-kDa γ-zein, which represents 12.2% of total zein message but accounts for only 4.6% of total zein protein. The 27-kDa γ-zein peak height is highly variable depending on the extraction conditions and matrix concentrations. Because the 27-kDa γ-zein is the most water-soluble zein, it is possible that most of the HABA has already precipitated by the time this zein precipitates, and thus, it is not well incorporated into the HABA matrix. The use of a more water-soluble matrix, such as sinapinic acid or α-cyanohydroxycinammic acid in conjunction with HABA, could potentially improve the signal of the 27-kDa γ-zein.

Comparison of MALDI-TOF MS Spectra from Different Inbred Varieties. Although the absolute levels of zeins cannot be concluded by the present procedures, the relative zein profiles by MALDI-TOF MS within different inbred varieties are consistent and can be compared on the basis of the same weight of ground kernel with normalization by an internal protein standard such as CA. A single representative spectrum from each of the inbred genotypes is shown (**Figure 4**). The spectra obtained from four individual kernels from each inbred were consistent.

Overall Zein Profiles. Large differences in peak intensity and molecular weight were observed among the spectra of the seven inbreds chosen for the study. In six of the seven inbreds examined, the 19-kDa α-zein subclass exhibited the most intense spectral peaks (B73, M14, Mo17, Oh43, W22, and W64A), whereas the 22-kDa α-zein spectral peaks were the most dominant peaks in the B37 line. M14 and Oh43 also have relatively intense 22-kDa α-zein peaks, but not in excess of the 19-kDa α-zein peaks.

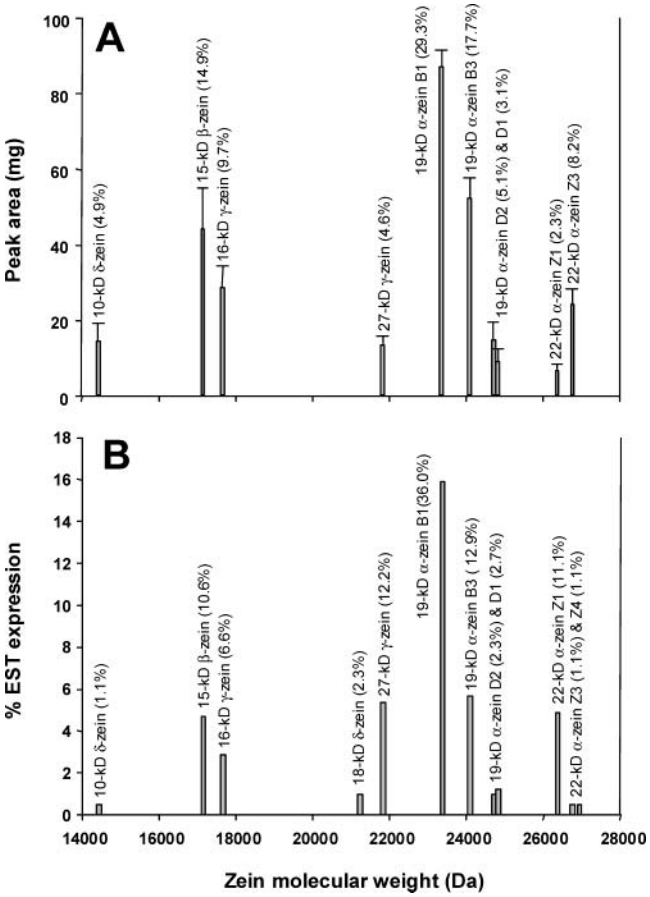

Figure 3. Relative abundance of zeins in B73. (**A**) Peak areas of zein peaks determined by MALDI-TOF MS plotted against their mass. Individual spectra from 12 B73 kernels were analyzed. Zein peaks from printed copies of the spectra were cut out, weighed, and averaged. The relative abundance (in parentheses) was calculated by dividing the area of each zein peak with the total area of all zein peaks. The HABA matrix concentration used in these spectra was 40 mM. (**B**) Zein transcript levels in B73 endosperm based on their percentage abundance among ESTs in endosperm-specific cDNA libraries plotted against mature protein molecular weight (*8*). Transcripts expressed below 0.5% are not plotted. The relative abundance (in parentheses) was calculated by dividing the percent EST of each zein peak with the total percent EST of all zeins shown.

α-Zeins. Typically, the most intense peak of the B73, Mo17, and W22 spectra is the lowest molecular weight 19-kDa α-zein. B37 and Oh43 lines exhibit an additional peak in close proximity to the 19-kDa α-zein peaks. This additional peak has a slightly lower molecular weight and appears as a doublet with an overall lower peak height than the single peaks observed in the majority of lines examined (**Figure 4**, solid arrows). In addition, in the B37 line, the highest molecular weight 19-kDa α-zein peak is the dominant peak among the 19-kDa α-zeins, whereas the middle peak of the 19-kDa α-zein subclass in M14, Oh43, and W64A is the highest among the subclass. In W22, a weaker middle peak of the 19-kDa α-zeins is distinctive. Similarly, small differences in the peak heights among the 22-kDa α-zein subclass are observed between the inbred lines.

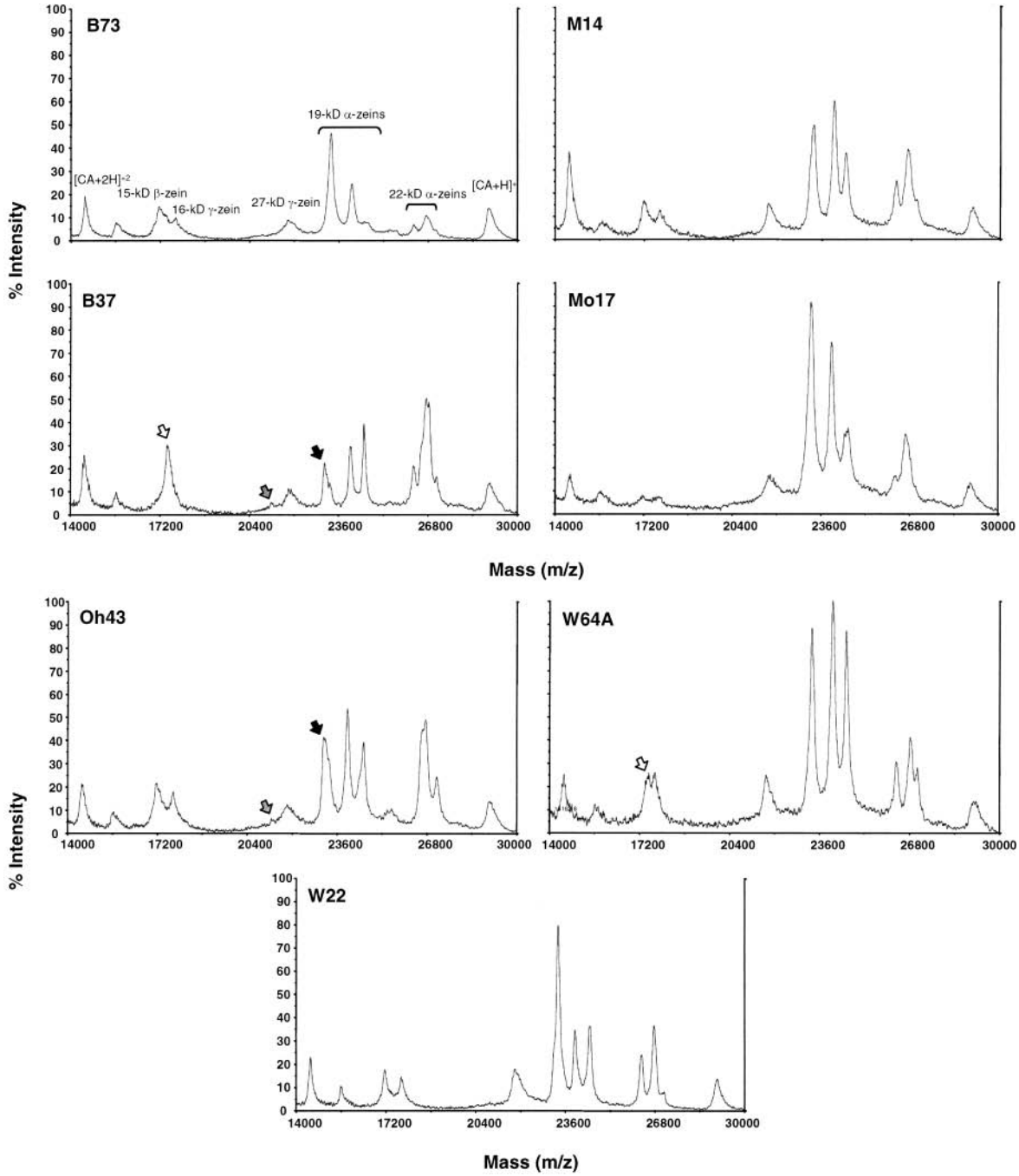

Figure 4. Zein profiles of inbred varieties by MALDI-TOF MS. Four kernels from each inbred varieties were analyzed under the same conditions. A representative spectrum from each inbred line is shown. Arrows point out variations observed within some zein subclasses between different inbred varieties. All of the spectra were normalized to the $[CA + H]^+$ internal standard.

β- and γ-Zeins. Most of the inbreds (B73, M14, Mo17, Oh43, and W22) have a 15-kDa β-zein peak at about ~17150 Da, not the 14458 determined from coding sequences (*8*). There is also a 16-kDa γ-zein peak at ~17750 Da and a 27-kDa γ-zein peak at ~21760 Da. B37 is unusual among the inbreds studied in that it has a single dominant peak at ~17500 Da (**Figure 4**, open arrows) and lacks the typical 15-kDa β-zein and 16-kDa γ-zein peaks. W64A also exhibits this ~17500-Da intermediate peak along with the 16-kDa γ-zein peak while also lacking the typical 15-kDa β-zein peak. A small quantity of the 17500-Da peak is observed in B73 and perhaps in some other inbreds, but the peak intensity is too low to be distinctive. The number of cysteine residues in the 17500-Da peak observed in B37, B73, and W64A has not been determined.

δ-Zeins. A very small peak at 21200 Da (**Figure 4**, shaded arrows) is observed in the B37 and Oh43 spectra but appears to not be present in the spectra from the other inbred lines. It corresponds to the molecular weight of 18-kDa δ-zein at 21220, which was not identified in B73 spectra. Derivatization experiments on B37 and Oh43 zein extracts are being performed to confirm the identity of the 21220-Da peak. The 10-kDa δ-zein, presumably at 14431 Da, is not detectable in these spectra, most likely due to its close proximity to the carbonic anhydrase standard $[CA + 2H]^{2+}$ at 14513 Da.

MALDI-TOF MS Analysis of Two *Opaque* Mutants. The MALDI-TOF MS tool is useful for examining the relative abundance of different zein classes within inbred lines. We utilized this tool to examine the zein profiles of two opaque

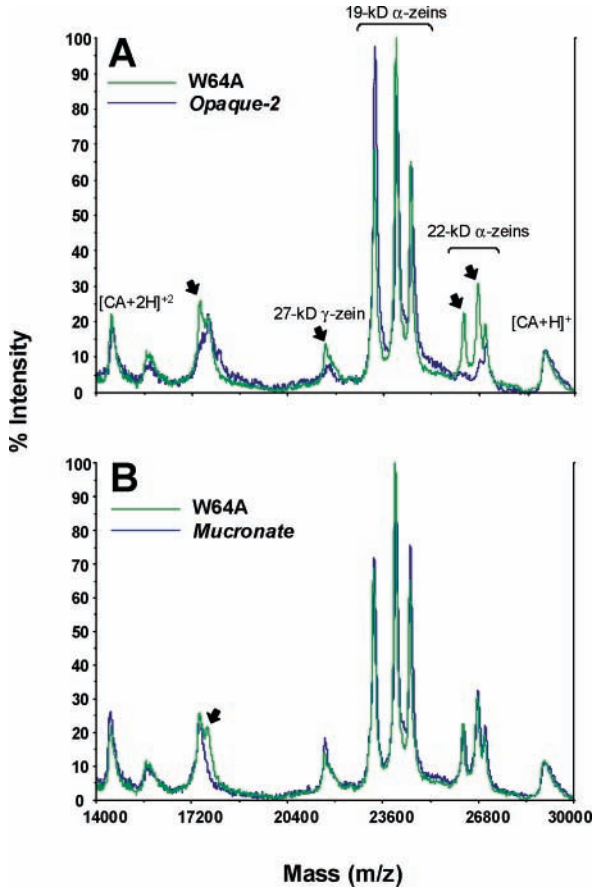

Figure 5. MALDI-TOF MS analysis of two *opaque* mutants. Overlaid spectral traces of zein profiles from wild-type (W64A) and *opaque-2* (**A**) or *mucronate* (**B**) mutant are shown. The mutants are in a W64A background. Arrows indicate the zein peaks that are missing or reduced in the mutants. The spectra were normalized according to the $[CA + H]^+$ internal standard.

mutants, *opaque-2* (*23*) and *mucronate* (*24*), as illustrated in **Figure 5**. These known mutants have alterations in the zein protein composition, and, in general, the kernels are relatively rich in lysine and tryptophan due to the reduction in their zein content. In addition to the alteration in zein accumulation, opaque phenotypes are associated with these mutants. Two of the 22-kDa α-zeins in *opaque-2*, which appear to show obvious reduction (**Figure 5A**), are consistent with earlier studies (*25*). It also appears that there are reductions in the protein peaks designated as the 27-kDa γ-zein and the 15-kDa β-zein (intermediate peak mentioned earlier). In the spectra obtained through analysis of the *mucronate* mutant (**Figure 5B**), the 16-kDa γ-zein at ~17759 Da is totally absent, with no apparent changes in the other zein peaks. These spectra demonstrate the ability of MALDI-TOF MS to qualitatively and semiquantitatively detect the zein changes in zein mutants and help to illustrate the utility of the MALDI-TOF MS as a tool for studying zein mutants.

Conclusion. MALDI-TOF MS allows for rapid characterization of unfractionated zein protein extracts with greater speed and accuracy than conventional gel-based methods. Analysis of proteins with MALDI-TOF MS produces spectra that provide a more accurate assessment of molecular masses and a better quantitation of relative abundance than can be obtained through analysis by SDS-PAGE. We present results based on the use of MALDI-TOF MS as a simple alternative method to conventional protein gel analysis for analyzing zeins derived from different inbred varieties and known zein mutants. This work suggests it might be possible to use this technology as a tool to utilize the variation observed among the zein proteins as a means for genotype identification. In addition, by examining segregating F1 hybrid kernels and their respective inbred parents, this method could also be used to investigate the molecular genetics of zein protein expression, which is normally difficult to study due to the complexity of their multigene families.

ABBREVIATIONS USED

MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; HABA, 2-(4-hydroxyphenylazo)benzoic acid; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EST, expressed sequence tag; ELISA, enzyme-linked immunosorbent assay; ACN, acetonitrile; DTT, dithiothreitol; IAA, iodoacetamide; CA, bovine erythrocyte carbonic anhydrase; TFA, trifluoroacetic acid.

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