JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Analysis of Zein by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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Matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) was used to analyze the protein composition of corn prolamine (zein). Mass spectra were obtained from commercial zein and zein extracted with aqueous 2-propanol and aqueous ethanol from consumer corn meal. For the commercial zein, three major zein fractions with *m*/*z* 26.8k, 24.1k, and 23.4k were clearly seen with two minor fractions (*m*/*z* 14.5k and 20.4k) also present. As compared with the results from sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), these three fractions were identified as α-zeins (24.1k and 23.4k combined as Z19; 26.8k as Z22). When extracted with 55% aqueous 2-propanol, three α-zein fractions with *m*/*z* 26.8k, 24.1k, and 23.4k were predominant. When extracted with ethanol, extraction temperature had an effect on the final products. When extracted with 75% aqueous ethanol at room temperature, α-zein and some 17−18k species were observed, whereas at 60 °C, a small amount of δ-zein was also present. Comparison of the MALDI/MS results with SDS−PAGE and gene sequence analysis shows that the MALDI/MS method is superior to SDS−PAGE in having higher resolution and mass accuracy.

KEYWORDS: Zein; molecular weight; matrix-assisted laser desorption/ionizatin (MALDI); mass spectrometry

INTRODUCTION

The alcohol soluble storage proteins (prolamines) of corn are a mixture of polypeptides that constitute about 50−60% of the total endosperm protein (*1*). According to the widely accepted nomenclature system developed by Esen, these proteins are classified as α-, β-, γ-, and δ-zeins on the basis of differences in solubility and sequence (*2*−*4*).

At present, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) has been used widely for characterization of the zein protein composition (*2*−*6*). It is a standard technique for the separation and characterization of proteins, providing measures of their crude molecular weights as well as their relative abundance. This method, however, has its limitations such as low mass accuracy and poor resolution. Reversed-phase high-performance liquid chromatography (RP-HPLC) has also been used (*7*), providing excellent resolution but giving the elution time instead of molecular weight.

According to Esen's SDS−PAGE results (see **Figure 1**) obtained using endosperm from an inbred K55 maize (*4*), α-zein (lane 1) is the most abundant (80% of total zein) and includes two protein groups, Z19 and Z22, with apparent molecular weights of 23.8k and 26.7k, respectively. β-Zein (lane 3) consists of a methionine-rich polypeptide of 17k and constitutes up to

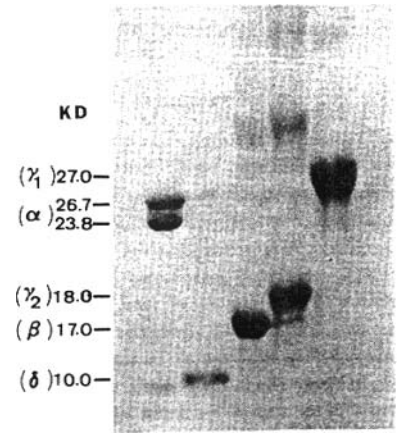

Figure 1. Various zein fractions and their subunit compositions as resolved by SDS−PAGE (reprinted with permission from ref *4*. Copyright 1990 Kluwer Academic Publishers). Esen used endosperm of inbred K55 for their extraction. The lanes represent different zein fractions based on their solubility in different solvents.

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10.1021/jf026133g CCC: $25.00
Published on Web 08/20/2003

Table 1. Comparison of MALDI/MS, SDS–PAGE, and Gene Sequence Analysis Results for Commercial Zein Compositions

	molecular weight				
name	SDS–PAGE[a]	SDS–PAGE[b]	MALDI/MS[b]	calculated[c]	difference[d]
22 kDa α-zein	26.7k	24k	26 838	26 359	479
19-kDa α-zein	23.8k	22k	23 362	23 359	3
			24 097	24 087	10
δ-zein	10k	10k	14 466	14 431	35

[a] Ref *4*. [b] Work in this paper. [c] Ref *15*. [d] The difference between the MALDI/MS and the calculated molecular mass from amino acid composition based on gene sequence (ref *15*).

10% of the total zein. γ-Zein is composed of two peptides: γ_1-zein (lane 5) with a molecular weight of 27k and γ_2-zein (lane 4) with a molecular weight of 18k. δ-Zein (lane 2) is a minor fraction and has a molecular weight of 10k.

With the recent development of new ionization methods, mass spectrometry (MS) has become an alternative method to characterize high molecular mass molecules and molecular composition. In particular, matrix-assisted laser desorption/ionization (MALDI) in combination with time-of-flight mass spectrometry (TOF-MS) has made it possible to study complex mixtures of large, thermally labile, nonvolatile biopolymers such as proteins with molecular masses of up to several hundred kilodaltons. MALDI/MS exhibits high sensitivity, high mass accuracy, and covers a wide mass range (from a few hundred daltons to several hundred kilodaltons).

MALDI/MS has recently been used for the analysis of complex cereal protein mixtures (*8*−*14*). For example, MALDI/MS was used in several studies (*8*, *9*) to characterize the subunits of wheat glutenin. Dworschak et al. (*9*) reported the use of MALDI/MS for assessing the composition and mass distribution of crude and partially purified wheat gluten prolamines without prior separation by HPLC. These studies show the feasibility of using MALDI/MS for the rapid analysis of complex protein mixtures. However, to date, the use of MS for characterization of plant storage protein is still somewhat limited.

Here, we report the use of MALDI/MS for assessing the composition and molecular mass of lab-extracted and commercial zein. The results are compared with SDS−PAGE results and those predicted by gene sequence analysis (*15*).

MATERIALS AND METHODS

IGA yellow enriched degerminated corn meal was obtained from IGA, Inc. (local grocery store). Commercial zein (regular grade F4000, from Freeman Industries, Inc., Tuckahoe, NY) was used without any treatment.

Extraction of Zein from Corn Meal. Twenty-five grams of corn meal was defatted by continuous extractions in a Soxhlet extractor with 150 mL of dry *n*-butanol for 6 h, washed with 50 mL of *n*-hexane, and air-dried for 2 days. Zein was extracted from 10 g of defatted corn meal with 45 mL of 55% (v/v) aqueous 2-propanol overnight at room temperature under gentle stirring. The extract, after filtering, was mixed with an equal volume of 2% NaCl aqueous solution and left overnight in a refrigerator to precipitate zein proteins. The precipitate obtained was filtered, rinsed with deionized water, and dried at room temperature.

Zein was also extracted with 75% ethanol/water from corn meal at room temperature and at 60 °C. The extraction procedures were similar to those for 2-propanol.

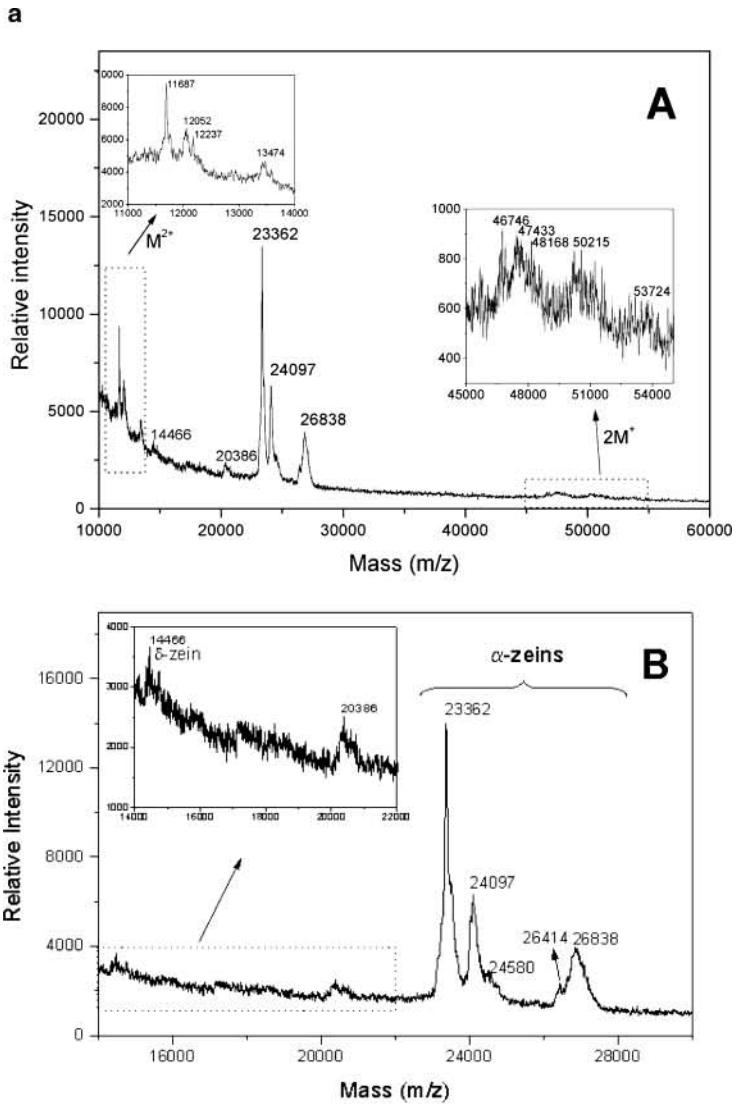
MALDI/MS Experiments. MALDI measurements were performed on a Voyager-DE STR system (Applied Biosystems, Inc., Foster City, CA), operating in positive ion linear mode. Ions formed by a pulsed UV laser (nitrogen laser, $\lambda = 337$ nm) were accelerated at 25 kV. The various samples of zein were dissolved in 55% (v/v) 2-propanol/water (concentration 5 mg/mL) with 5% formic acid to promote dissolution. The supernatant after centrifugation was diluted 10 times with 10 mg/mL matrix materials; 2,5-dihydroxyl benzoic acid (DHB, Sigma Chemical Co., St. Louis, MO) was used as matrix material. 2-(4-Hydroxy-phenylazo)benzoic acid (Aldrich Chemical Co., Milwaukee, WI) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Fluka Chemie AG, Milwaukee, WI) were also used as matrix materials, but the results showed that they were not as good as DHB in the analysis of zein in terms of signal-to-noise ratio and reproducibility. All scans were smoothed using Origin 5.0.

Gel Electrophoresis. Zein was resuspended in 55% 2-propanol to a concentration of 2.5 mg/mL. Samples were then centrifuged at 14 000*g*, and the supernatant was retained. The samples were then dried under dry argon and resuspended in 50 mM Tris, pH 6.8, 100 mM dithiothreitol (DTT; for the nonreduced samples, DTT was omitted), 2% SDS, 0.1% (w/v) bromophenol blue, and 10% glycerol to a final zein concentration of 7.5 mg/mL. The samples were then incubated at 50 °C for 10 min and loaded onto the gel. SDS−PAGE of commercial and lab-extracted zein was carried out in a SDS−12% polyacrylamide gel (SDS−Polyacrylamide Gel System, Bio-Rad Laboratories, Hercules, CA) with a running buffer containing 49 mM Tris, 384 mM glycine, and 0.1% (w/v) SDS, pH 8.5. Coomassie blue staining was used for detection of the zein proteins.

RESULTS AND DISCUSSION

MALDI/MS and SDS−PAGE Results for Commercial Zein. Figure 2aA is a typical MALDI/MS spectrum of the commercial zein. Besides molecular ion peaks, there are doubly charged peaks and dimer peaks (see the insets). **Figure 2aB** shows the major molecular ion peaks with their assignment to various zein fractions based on the similarity of molecular weight and method of extraction according to Esen's nomenclature (*2*−*4*). α-Zein is dominant in the commercial zein with two small peaks at *m*/*z* 14 466 and 20 386. The measured α-zein molecular weights are listed in **Table 1**.

In the literature, SDS−PAGE results usually divide α-zein into two groups based on their migration (Z19 and Z22). However, the apparent molecular mass of the peptides was often different in the various reports because of the use of different gel systems, standard proteins, and corn varieties. Apparent molecular masses of 18−24k for Z19 and 21−26k for Z22 have been reported by different authors (*7*). Our SDS−PAGE shows two bands at 22k Da and 24k Da (see **Figure 2b**). Actually, analysis by isoelectric focusing (*16*) and RP-HPLC (*7*, *17*−*18*) indicated α-zein to be a mixture of a large number of proteins. For example, Wilson showed at least 15 components in α-zein by RP-HPLC serial analysis (*7*). From the MALDI/MS results (see **Figure 2aB**), we attribute three broad peaks to α-zein. Those attributed to Z19 α-zein consist of two broad peaks at *m*/*z* 23 362 and 24 097 while the peak at 26 838 is attributed to Z22 α-zein. The breadth and shoulder peaks at *m*/*z* 26 414 and 24 580 suggest that each peak includes multiple components, in agreement with the large number of proteins in α-zein. The three main α-zein components agree well in molecular weight with the results from Woo et al. (*15*). On the basis of an analysis of endosperm expressed sequence tag libraries, they identified nine α-zein coding sequences with the three most abundant



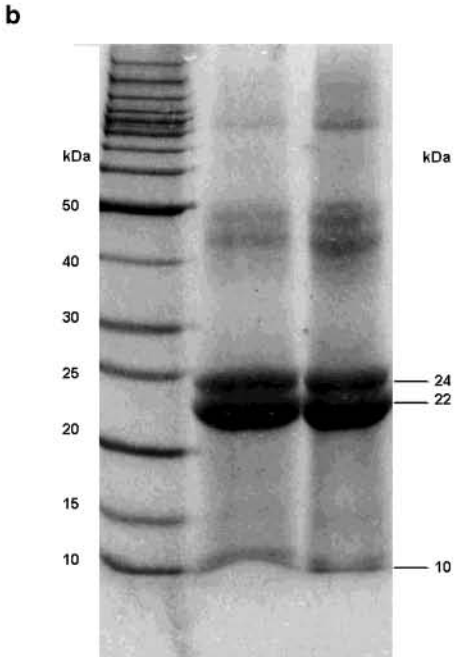


Figure 2. (a) MALDI mass spectrum of commercial zein (Freeman regular grade). (A) The upper left inset shows the doubly charged peaks, and the lower right inset shows the dimer peaks. (B) An expanded spectrum of A with the inset showing the minor zein fractions. (b) SDS–PAGE of commercial zein run with (lane 2) and without (lane 3) DTT. Lane 1 is the molecular weight standard ladder.

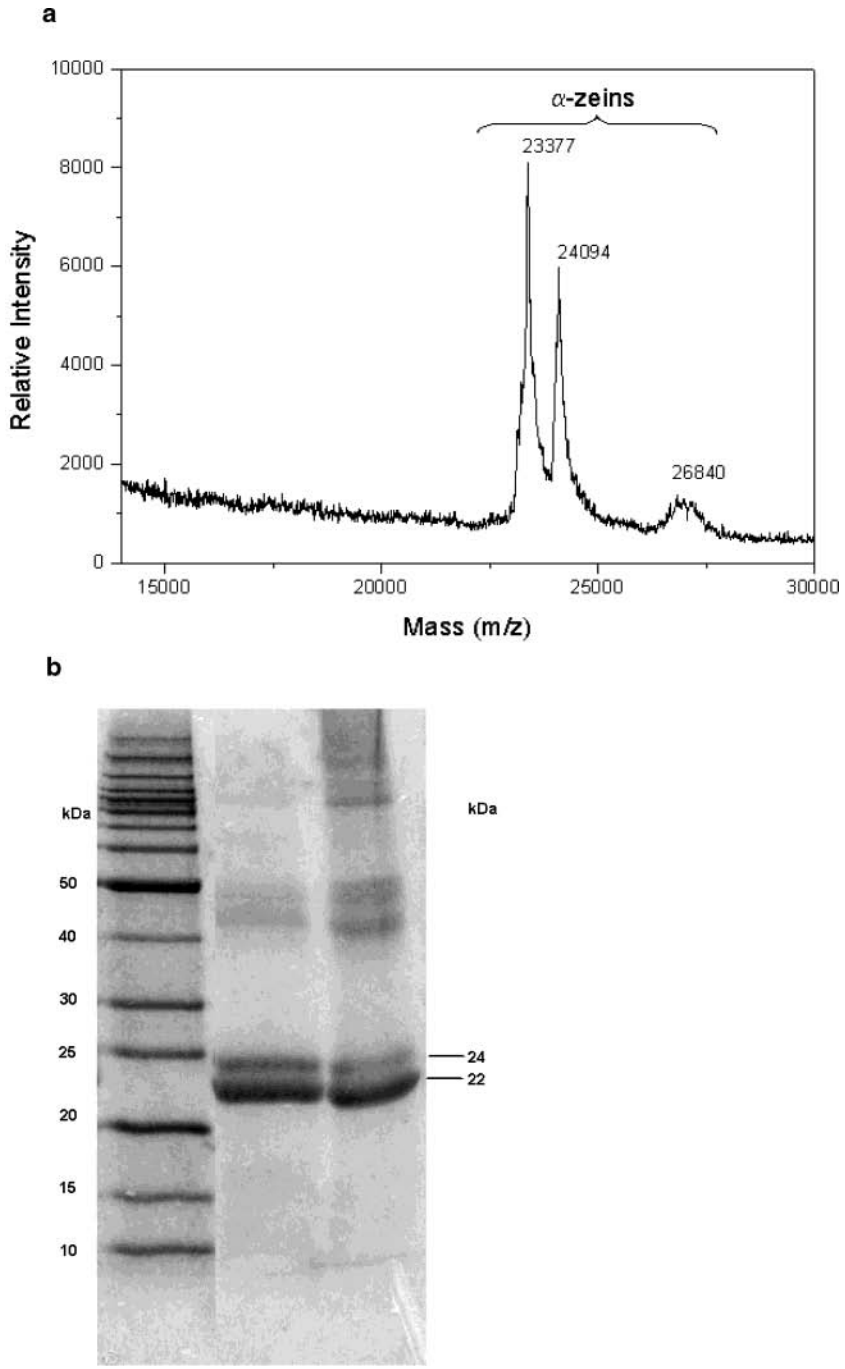


Figure 3. (a) MALDI spectrum of 55% 2-propanol extracted zein. (b) SDS–PAGE of 55% 2-propanol extracted zein run with (lane 2) and without (lane 3) DTT. Lane 1 is the molecular weight standard ladder.

being 19k Da B1 (calculated molecular weight 23 359), 19k Da B3 (calculated molecular weight 24 087), and 22k Da Z1 (calculated molecular weight 26 359).

The peak at *m*/*z* 14 466 in MALDI/MS can be assigned to δ-zein. The calculated molecular weight for δ-zein from sequence analysis is 14 431, very close to the MALDI result. δ-Zein was observed at 10k in our SDS−PAGE runs (see **Figure 2b**). Wilson (*19*) also saw δ-zein at 10k in commercial zein (Freeman) runs on SDS−PAGE. δ-Zein was observed in our MALDI/MS spectra run without reducing agents.

The species at *m*/*z* 20 386 in MALDI/MS was not clearly observed in our SDS−PAGE, although there were suggestions of low density bands at ca. 16k and 18k. These peaks could be due to 18k δ-zein, which has a calculated molecular weight of 21 220, or to 27k γ-zein, with a calculated molecular weight of 21 822 (*15*). Another possibility is 18k globulin, which has a calculated molecular weight of 20 299 (*15*). To correctly assign this species, the zein components will need to be further

Table 2. Comparison of the Molecular Weights Obtained from MALDI/MS for Commercial Zein and 55% 2-Propanol Extracted Zein

	molecular weight from MALDI/MS		
zein	commercial zein	zein extracted with 55% 2-propanol	difference[a]
α-zein	23 362	23 377	15
	24 097	24 094	–3
	26 838	26 840	2

[a] The difference between molecular weights of 55% 2-propanol extracted zein and commercial zein.

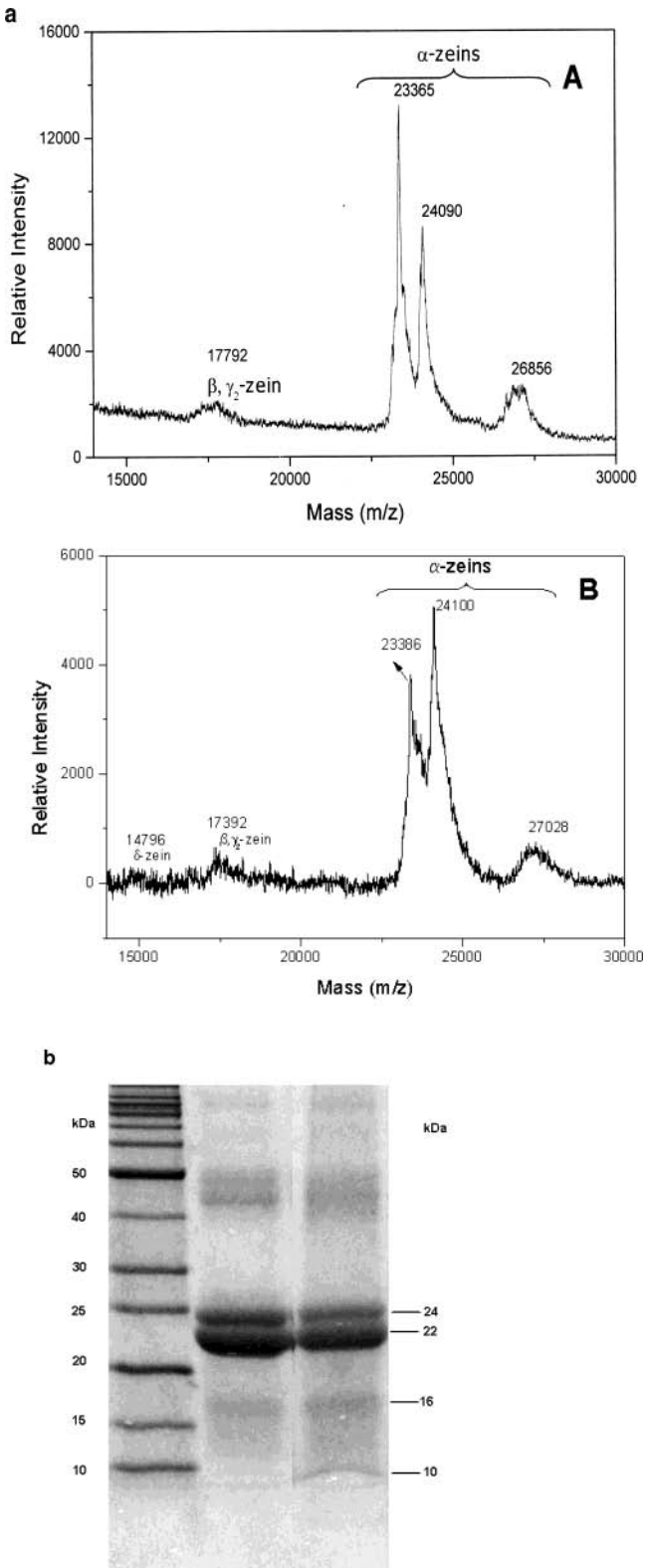


Figure 4. (a) MALDI mass spectrum of 75% ethanol extracted zein at room temperature (A) and 60 °C (B). (b) SDS–PAGE of 75% ethanol extracted zein at room temperature (lane 2) and 60 °C (lane 3) without adding reducing agent. Lane 1 is the molecular weight standard ladder.

separated, concentrated, and run on MALDI/MS and SDS−PAGE followed by comparison with the calculated molecular weight from gene sequence analysis.

Comparison of molecular weights from MALDI/MS spectra with those predicted from gene sequencing for the three major zein components (*15*) shows differences within 1.8% (**Table 1**). The correspondence is remarkably good. There are several factors that may account for the mass differences between MALDI/MS and the predicted values. First, the measurement accuracy of mass decreases for broad peaks. We found differences up to 100 Da between repeat measurements of the same sample, which may be due to peak shifts due to software smoothing. In addition, the minor components determined by the gene sequencing will contribute to the MALDI/MS peaks, resulting in some shift in peak position. We also need to account for the errors from the gene sequence analysis method itself.

MALDI/MS and SDS−PAGE Results for 2-Propanol Extracted Zein. Figure 3a shows the MALDI/MS spectra of zein extracted with 55% 2-propanol from corn meal; α-zein is dominant. A comparison between the molecular weights obtained from MALDI/MS of 2-propanol extracted α-zein and commercial zein is listed in **Table 2**.

The SDS−PAGE run of the 55% 2-propanol extracted zein (**Figure 3b**, lane 2) shows the two α-zein bands (Z19 and Z22), but no other zein components were observed even with the reducing agent (**Figure 3b**, lane 3); this is consistent with our MALDI/MS observation. Here and in **Figure 2b**, the lanes without reducing agent (lane 3) show the presence of a greater relative concentration of dimers and larger aggregates.

The observation of 14 466 and 20 386 peaks in the commercial zein (**Figure 2a**) but not in our 55% 2-propanol extracted zein (**Figure 3a**) is believed to be mainly due to the difference in extraction process and, possibly, the corn used. This also demonstrates the ability of the MALDI/MS method, for instance, to analyze zein from different corn varieties and extraction processes.

We notice that the molecular weights corresponding to the same peak for the commercial zein and for 2-propanol extracted zein are only slightly different (see **Table 2**). As mentioned above, this is mainly because all of the peaks are actually broad bands making it difficult to determine the exact peak values and also because of the possible differences in smoothing, with the values thus being surprising close.

MALDI/MS and SDS−PAGE Results for Ethanol-Extracted Zein. Extracted zein is a complex protein mixture. Its composition and relative proportion of the different fractions vary depending on extraction conditions. M. Cheryan is conducting research on reducing the cost of zein production utilizing membrane technology to separate, isolate, and purify zein from ethanolic extracts and to recycle the ethanol used as solvent without substantial evaporation (*20*, *21*). Thus, we extracted zein from corn meal with an ethanol−water solvent system at two different temperatures and used MALDI/MS to analyze the products.

When zein was extracted with 75% (v/v) ethanol/water at room temperature (**Figure 4aA**), besides α-zein, we also obtained some 17−18k species, which could be assigned, based on the similarity in molecular weight with those predicted by gene sequence analysis (*15*), to β-zein (calculated molecular weight 17 458) or γ_2-zein (calculated molecular weight 17 663). A 16k zein peptide was observed on an SDS−PAGE run (**Figure 4b**, lane 2), which confirms the MALDI/MS result. Further work needs to be done to know whether the 17−18k peak in the MALDI/MS spectrum is β-zein or γ_2-zein.

Figure 4aB shows the MALDI/MS result of zein extracted with 75% ethanol/water at 60 °C. As compared with the room temperature extraction products, δ-zein was observed at *m*/*z* 14 796 only at elevated temperature. In the SDS−PAGE, the 10k δ-zein is present in product extracted at 60 °C (lane 3) but absent in that extracted at room temperature (lane 2), which is

consistent with the MALDI/MS result. Here, again, MALDI/M shows its ability to monitor the products from different extraction conditions.

CONCLUSIONS

MALDI/MS was used to study the composition and molecular mass of both lab-extracted and commercial zein. Three major components were assigned to α-zeins. Further work needs to be done in order to correctly assign the minor fractions. The results show that as compared with the traditional SDS−PAGE method, determination of the composition of zein with MALDI/MS has a higher mass accuracy, resolution (peak separation), and versatility. The resolution, however, is lower than that of RP-HPLC.

ACKNOWLEDGMENT

We thank Prof. Esen and Kluwer Academic Publishers for kindly allowing us to use their figure (**Figure 1**).

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Received for review November 15, 2002. Revised manuscript received July 2, 2003. Accepted July 17, 2003. This research was supported, in part, by the Illinois Corn Marketing Board, the Illinois Council for Food and Agricultural Research, and the Illinois Agricultural Experiment Station. High-resolution mass spectra were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, supported in part by a grant from the National Institute of General Medical Sciences (GM 27029). The Voyager mass spectrometer was purchased, in part, with a grant from the Division of Research Resources, National Institutes of Health (RR11966).

JF026133G