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MALDI-TOF MASS SPECTROMETRIC ANALYSIS OF ZEINS EXTRACTED FROM MAIZE SEEDS

Raluca Ştefănescu* and Sabina Băncilă

Faculty of Chemistry, "Al. I. Cuza" University of Iasi, 11 Carol I Bd, Iasi 700506, Romania

Abstract: We report the mass spectrometric analysis of zeins extracted from degreased maize flour with either an aqueous 70% ethanol solution or 60% acetonitrile containing 10 mM dithiothreitol. The analysis was performed on a MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid as matrix. The method allowed the detection of α-, β- and γ-zeins, but not δ-zeins and was used for the analysis of zein content in the maize inbred KWS 3381 and in commercial flour at different flour particles sizes: between 710 μm and 1.0 mm, between 250 μm and 355 μm and smaller than 100 μm.

***Keywords*:** MALDI-TOF; prolamins; *Zea mays*; zeins

Introduction

Zeins belong to the storage proteins named prolamins, which contain a high number of proline and glutamine units. Prolamins are located in the endosperm of many cereal seeds and are soluble in 70% alcohol.[1-3] There are four groups of zeins: α-zeins, β-zeins, γ-zeins and δ-zeins. According to the electrophoretic migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the first group belongs to α-zeins, which are

* Raluca Ştefănescu, *e-mail:* rallstef@gmail.com

comprised of 19 kDa α-zeins and 22 kDa α-zeins, the second one, β-zeins, is represented by 14 kDa β-zeins, the third one contains γ-zeins that are comprised of 16 kDa and 27 kDa γ-zeins and, finally, δ-zeins are represented by 10 kDa δ-zein.[1]

Table 1. List of the GenBank and Uniprot accession numbers for the zein proteins identified in the B73 maize inbred by Woo, Y.-M. and collaborators and comparison between the calculated and observed masses of the zeins from the spectra as reported by Adams W.R. and collaborators.

Zein type	GenBank accession no.	Uniprot accession no.	Total no. of amino acids* (no. of cysteine residues)	$[M+H]^+_{calc.}$**	$[M+H]^+_{exp.}$***	No. of prolamine specific amino acids	
						P	Q
10-kDa δ-zein	AF371266	Q41881	129 (5)	14432	14432	20	15
15-kDa β- zein	AF371264	Q946W0	158 (7)	17148	17125	12	25
16-kDa γ- zein	AF371262	Q548E8	164 (12)	17751	17714	25	31
18-kDa δ- zein	AF371265	Q946V9	190 (3)	21222	-	35	14
27-kDa γ- zein	AF371261	Q548E9	204 (15)	21823	21793	51	30
19-kDa α- zein B1	AF371269	Q946V6	213 (2)	23360	23318	23	41
19-kDa α- zein B3	AF371271	Q548E6	219 (2)	24088	24069	22	43
19-kDa α- zein D2	AF371268	Q946V7	220 (1)	24707	24515	18	47
19-kDa α- zein D1	AF371267	Q946V8	222 (1)	24819	24644	19	45
22-kDa α-zein Z1	AF371274	Q9SBC4	242 (1)	26360	26308	22	50
22-kDa α-zein Z5	AF371277	Q9SYT3	245 (1)	26711	-	19	50
22-kDa α-zein Z3	AF371275	O48966	245 (1)	26752	26741	22	51
22-kDa α-zein Z4	AF371276	Q946V4	246 (1)	26924	-	21	53
19-kDa α-zein B2	AF371270	Q548E7	246 (2)	27129	-	22	47

*without the signal sequence
**considering all cysteine residues reduced
***reported by Adams W.R. and collaborators

Adams W.R. and the collaborators reported the identification of the zeins from the maize inbred B73 based on the molecular weights calculated for the amino acid sequences that have the signal peptide removed and which are found at the GenBank accession numbers reported by Woo, Y.-M. *et al.* and the molecular weights obtained by MALDI-TOF mass spectrometric analysis (see Table 1).[4-6] Moreover, the authors treated the zein extracts with iodoacetamide in order to alkylate the cysteine residues after the disulfide bridges reduction with dithiothreitol. The number of modified cysteine residues was estimated by comparing the mass spectra of the alkylated zeins with the mass spectra of the underivatized zeins. The peak assignment to α-, β-, γ- or δ-zeins was possible due to the different content in cysteine residues of the sequences of zeins (Table 1).

Most of the proteins contain a number of lysine and arginine amino acid residues that allows the enzymatic cleavage of the protein using trypsin. In the case of the zeins, due to the low content in the amino acids lysine and arginine, a proteolytic cleavage using trypsin followed by the mass spectrometric analysis of resulted fragments will lead to difficulties in sequence assignment caused by the high length of these fragments (Table 2). The endoprotease Glu-C (from *Staphylococcus aureus* strain V8) is cleaving the peptide bonds C-terminally at glutamic or aspartic acid if the reaction is carried out in phosphate buffer saline (PBS). However, the number of these two specific amino acids is very low in all zeins. Alpha-chymotrypsin is a proteolytic enzyme which cleaves at the carboxyl end of tyrosine, phenylalanine, tryptophan, and leucine. Hydrolysis may also occur on the C-terminal side of methionine, isoleucine, serine, threonine, valine, histidine, glycine, and alanine. Due to the fact of huge number of cleavage

sites, a plenty of very short sequences would be generated after cleavage with alpha-chymotrypsin.

Table 2. The number of cleavage sites of zeins in the presence of trypsin, Glu-C or alpha-chymotrypsin identified by Woo Y.-M and collaborators.[6]

Zein type	Trypsin cleavage sites		Glu-C cleavage sites		Alpha-chymotrypsin cleavage sites					
	K	R	D	E	Y	F	W	L	M	I
10-kDa δ-zein	0	0	1	0	1	5	0	15	29	3
15-kDa β- zein	0	5	1	3	12	1	0	16	18	1
16-kDa γ- zein	0	3	0	3	8	7	1	14	3	1
18-kDa δ- zein	1	0	1	0	2	5	2	13	48	10
27-kDa γ- zein	0	5	0	2	4	2	0	19	1	4
19-kDa α- zein B1	0	2	0	1	8	13	0	42	0	9
19-kDa α- zein B3	0	3	1	1	8	12	0	43	0	12
19-kDa α- zein D2	0	5	1	2	9	13	0	39	1	12
19-kDa α- zein D1	0	3	0	1	10	14	0	38	1	12
22-kDa α-zein Z1	0	2	0	1	8	9	0	44	4	8
22-kDa α-zein Z5	0	3	1	2	8	9	0	44	3	12
22-kDa α-zein Z3	0	3	0	1	7	9	0	42	4	12
22-kDa α-zein Z4	0	2	0	2	8	8	1	44	4	12
19-kDa α-zein B2	0	4	1	1	9	13	0	52	1	10

In the present study we identified the signals observed in the mass spectra acquired for the zein extracts from maize inbred KWS 3381 with the corresponding molecular weights for the singly charged ions of the zein calculated based on primary sequences available at GenBank and Uniprot, which are determined for the maize inbred B73 by Woo, Y.-M. and collaborators.

Results and Discussion

Using MALDI-TOF mass spectrometry, we first analyzed the zeins extracted with 70% ethanol in water from the maize inbred KWS 3381 flour with the particles size between 710 μm and 1.0 mm. The mass spectrum shown in Figure 1 displays peaks at m/z 23340, 24007 and 17731 that are assigned to the singly charged ions of the 19 kDa α-zein B1, 19 kDa α-zein B3 and 16 kDa γ-zein, respectively, and peaks at m/z 11670 and 8868 that correspond to the doubly charged ions of the 19 kDa α-zein B1 and 16 kDa γ-zein.

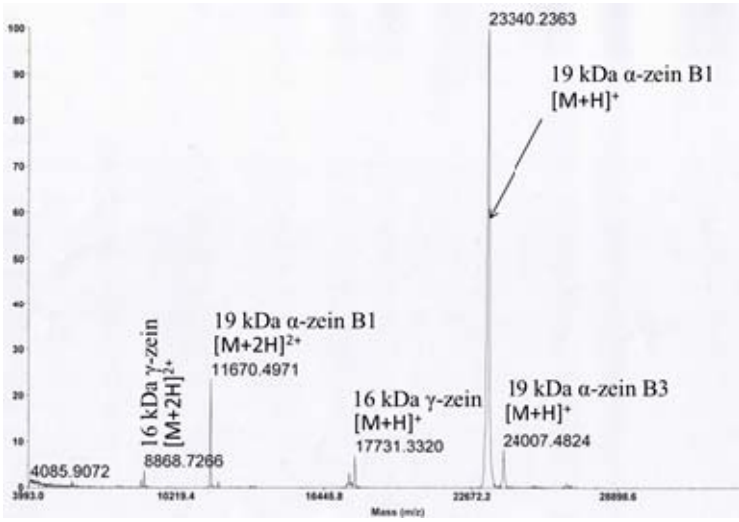

Figure 1. MALDI-TOF mass spectrum of the zeins from the maize inbred KWS 3381 flour having the particle size between 710 μm and 1.0 mm with 70% ethanol in water. The zeins were extracted by ultrasonication for 30 minutes in an ultrasonic water bath.

Figure 2 displays the mass spectrum acquired for the zeins extracted with 70% ethanol from the commercial maize flour that has the size of the particles between 710 μm and 1.0 mm. Based on the m/z values of the peaks present in the mass spectrum, three characteristic signals 19 kDa α-zein B1 (at m/z 23356), the 19 kDa α-zein B3 (at m/z 24035) and a 22 kDa α-zein (at m/z 26757) were identified.

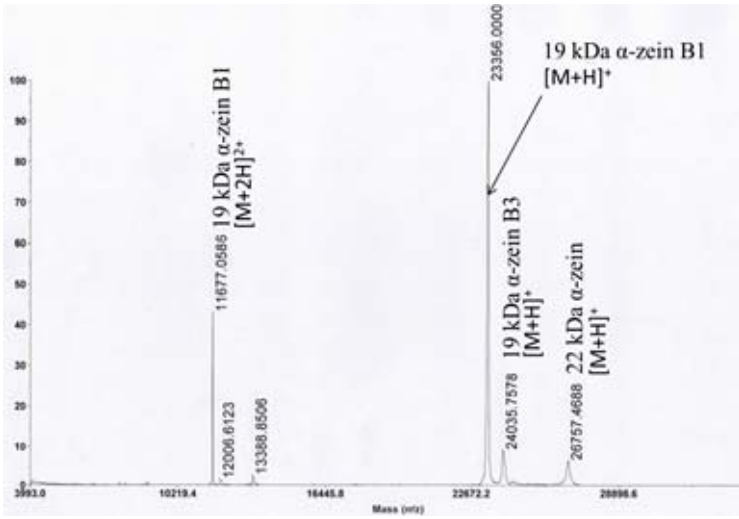

Figure 2. MALDI-TOF mass spectrum of the zeins extracted from commercial maize flour having the particle size between 710 µm and 1.0 mm, with 70% ethanol in water for 30 minutes using an ultrasound bath.

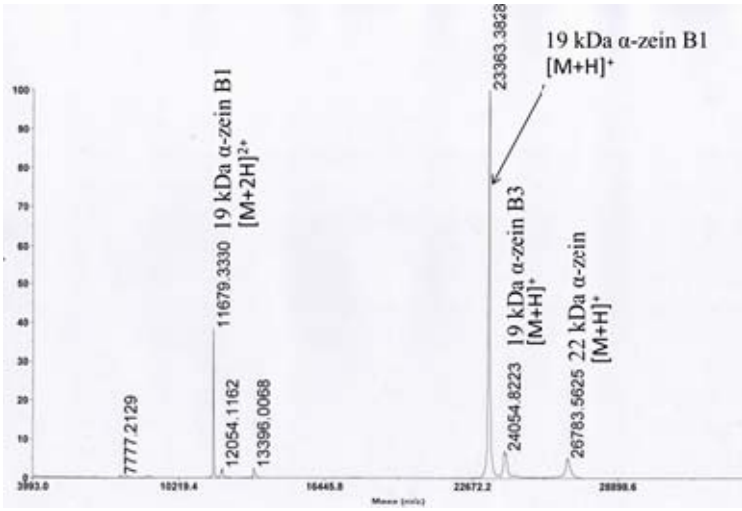

Figure 3. MALDI-TOF mass spectrum of the zeins extracted from commercial maize flour having the particle size between 250 µm and 355 µm with 70% ethanol in water for 30 minutes using an ultrasound bath.

Similarly, the mass spectrum of extracted proteins in 70% ethanol from commercial flour with particle size between 250 µm and 355 µm,

displays two signals at 19 kDa (α-zein B1 at m/z 23363 and α-zein B3 at m/z 24054) and one signal at 22 kDa α-zein (at m/z 26783) (see Figure 3).

Figure 4 shows the MALDI-TOF mass spectrum of the zeins extracted with 60% acetonitrile in water and 10 mM DTT from the maize inbred KWS 3381 that has the particle size between 710 μm and 1.0 mm. In addition to previous observed signals from above investigated extracts, 19 kDa α-zein B1 (at m/z 23345), the 19kDa α-zein B3 (at m/z 24014) and a 22 kDa α-zein (at m/z 26722), two supplementary signals 15 kDa β-zein (at m/z 17453) and the 16 kDa γ-zein (at m/z 17740) were noticed in the mass spectrum.

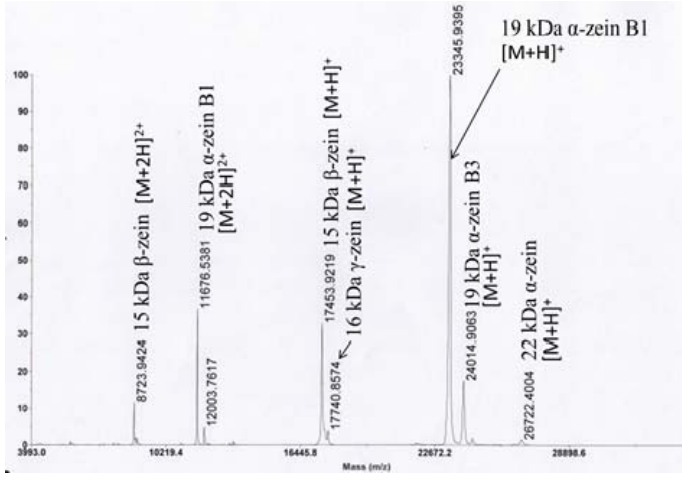

Figure 4. MALDI-TOF mass spectrum of zeins extracted from the maize inbred KWS 3381 flour, having the particle size between 710 μm and 1.0 mm, with 60% acetonitrile in water and 10 mM DTT for 60 minutes at 60°C.

The mass spectrum depicted in Figure 5 contains peaks that correspond to the zeins extracted with 60% acetonitrile and 10 mM DTT from the commercial flour with particle size between 710 μm and 1.0 mm. The peaks at m/z 23351, 24045 and 26751 were assigned to the singly charged ions of the 19 kDa α-zein B1, 19 kDa α-zein B3 and a 22 kDa

α-zein, respectively. The peaks at m/z 21847 and at m/z 17737 correspond to the 27 kDa γ-zein and to the 16 kDa γ-zein while the peaks at m/z 17443 and m/z 17143 correspond to the 15 kDa β-zein.

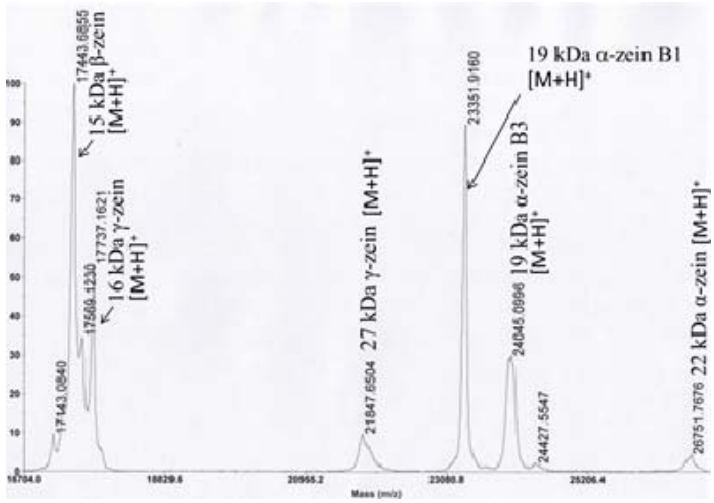

Figure 5. MALDI-TOF mass spectrum of the zeins extracted from commercial maize flour having the particle size between 710 μm and 1.0 mm, with 60% acetonitrile in water and 10 mM DTT for 60 minutes at 60°C.

The difference between the sample preparation for the mass spectrum presented in the Figure 5 and the mass spectrum presented in the Figure 6 consists only in that in the later the size of the commercial flour is between 250 μm and 355 μm. The results show that the peak corresponding to the 15 kDa β-zein and the 16 kDa γ-zein is less abundant and the peak assigned to the 27 kDa γ-zein is missing.

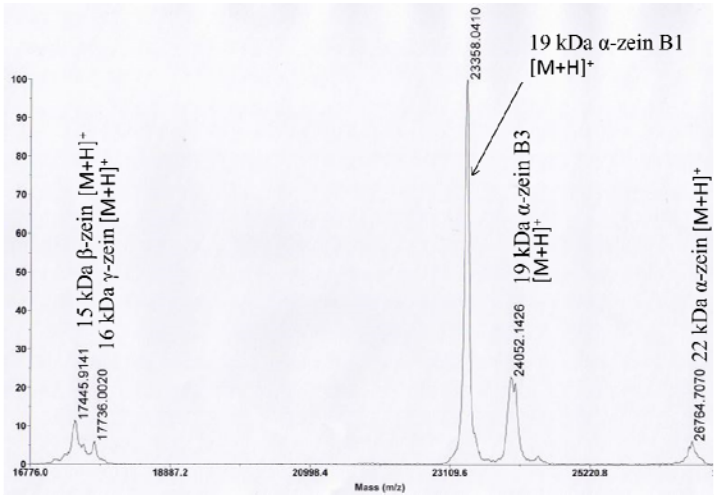

Figure 6. MALDI-TOF mass spectrum of the zeins extracted from commercial maize flour having the particle size between 250 μm and 355 μm with 60% acetonitrile in water and 10 mM DTT for 60 minutes at 60°C.

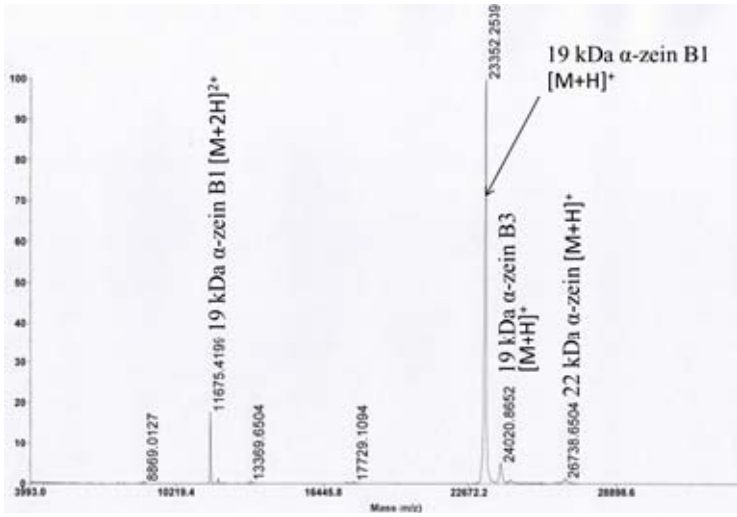

Figure 7. MALDI-TOF mass spectrum of the zeins extracted from the maize inbred KWS 3381, having the particle size 100 μm, with 70% ethanol in water for 30 minutes using an ultrasound bath.

In the Figures 7 and 8, the mass spectra of the zein extracted with ethanol 70% and acetonitrile 60% containing 10 mM DTT respectively from the maize inbred KWS 3381 that has the flour particle size smaller than 100 μm are presented.

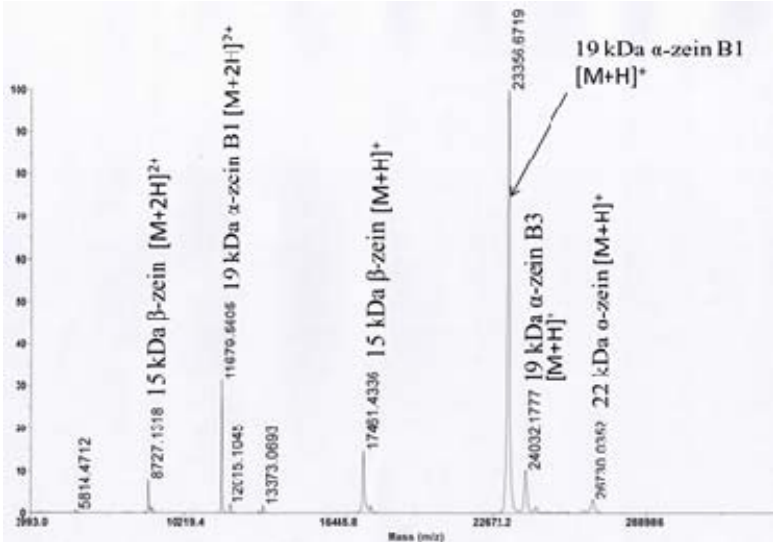

Figure 8. MALDI-TOF mass spectrum of the zeins extracted from the maize inbred KWS 3381flour, having the particle size smaller than 100 μm, with 60% acetonitrile in water and 10 mM DTT for 60 minutes at 60 °C.

In contrast to the other studies reporting the analysis by MALDI-TOF MS of zeins extracted from degreased maize flour using the matrices 2,5-dihydroxyl benzoic acid (DHB) or 2-(4-hydroxyphenylazo)benzoic acid (HABA), in the present study we employed the matrix α-cyano-4-hydroxycinnamic acid.[4,5] This matrix allowed the detection in the mass spectra of the 19 kDa α-zeins B1 and B3, a 22 kDa α-zein, the 27 kDa and the 16 kDa γ-zeins and the 15 kDa β-zein.

The extraction of the zeins with 70% ethanol led to the identification in the mass spectra of the 19 kDa α-zeins B1 and B3 and of a 22 kDa α-zein. The extraction with 60% acetonitrile and 10 mM dithiothreitol allowed the extraction of the 27 kDa γ-zein and the 15 kDa β-zein in addition to the zeins extracted in 70% ethanol.

Experimental

Materials

Maize seeds, inbred KWS 3381, were obtained from the company KWS (Germany). Commercial flour was obtained from a local grocery store. Petroleum ether and ethanol were purchased from Merck, acetonitrile, HPLC grade, and the matrix, α-cyano-4-hydroxycinnamic acid, were from Sigma-Aldrich. The trifluoroacetic acid, peptide synthesis grade, was from Scharlab. All solutions were prepared using deionized water from a MilliQ® Integral 3 system (Merck, Bucharest, Romania).

Preparation of the flour for zein extraction

Maize seeds from the inbred KWS 3381 were ground with a tripod portable mill (MB03, 1500 rpm, produced by IPEE, Romania). An amount of 20 g of the resulting flour was defatted with petroleum ether for 5 hours using a Soxhlet extractor. The flour was allowed to dry for 24 hours in a laboratory oven at 100°C. A sieve shaker (Retsch, Germany) was employed for selecting the flour with particles smaller than 710 μm. This flour was further ground using a laboratory electric mill (SAMAP F100, Andolsheim, France) until particles with the size lower than 100 μm were obtained.

20 g of commercial flour was defatted and dried as described above. For separating the flour particles with different sizes, sieves of 1.4 mm, 1 mm, 710 μm, 500 μm, 355 μm, 250 μm, 200 μm, 180 μm and 100 μm were employed using a sieve shaker.

Zein extraction

The extraction of the zeins from the maize flours was performed using two methods. In the first method, 150 mg of defatted flour that has selected particle size was mixed with 1.5 mL 70% ethanol in water for 30

minutes in an ultrasound bath. The time necessary for the extraction was established in the work reported by Bancila S. et *al.* and by Drochioiu G. et *al.*.[7,8] The samples were centrifuged for 10 minutes at 16,000 rpm using a Hettich Mikro 22R centrifuge (from Andreas Hettich GmbH&Co. KG, Tuttlingen, Germany). In the second method, 50 mg of defatted flour that has a selected particle size was added to 2.5 mL 60% acetonitrile, 10 mM dithiothreitol in water and was kept at 60 °C while mixing at every 15 minutes. After 60 minutes, the sample was centrifuged for 10 minutes at 16,000 rpm. The extraction of the samples was performed prior to their application on the target plate for the analysis by MALDI-TOF mass spectrometry.

Mass spectrometric analysis

For MALDI-TOF mass spectrometric analysis, a saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile: 0.1% trifluoroacetic acid in water (2:1 v/v) was prepared. The matrix solution was kept in an ultrasound bath for 15 minutes and centrifuged for 1 minute at 6000 rpm with a SPROUT® centrifuge commercialized by Heathrow Scientific, Illinois, USA. The zein extract prepared by either method 1 or method 2 was diluted tenfold and 20 fold with the supernatant of the matrix solution. A volume of 1 μL of each sample-matrix solution was placed on a 384-sample spots target plate and allowed to dry for 1 hour. The samples were analyzed in linear mode with the AB SCIEX TOF/TOF 5800 mass spectrometer equipped with a Nd:YAG laser that operates at 349 nm. External calibration was performed using the average mass of the singly charged ions of bovine insulin (5734.59), thioredoxin (11674.48) and apomyoglobin (16952.56).

Conclusions

In the present study, MALDI-TOF mass spectrometry was employed for rapid analysis of zeins extracted with solutions containing 70% ethanol in water or 60% acetonitrile in water containing 10 mM dithiothreitol. The matrix α-cyano-4-hydroxycinnamic acid provided a good detection of α-, β- and γ-zeins. Amino acid sequence assignment was possible due to previous identification of zeins reported by Adams *et al.* which was based on the comparison between experimental and calculated molecular masses for the zein amino acid sequences determined by Woo et *al.* and on the calculation of the number of cysteine residues from the mass spectra of alkylated and underivatized zeins.

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References

1. Shewry, P.R.; Halford, N. G. Cereal seed storage proteins: structures, properties and role in grain utilization. *J. Exp. Bot.* **2002**, *53*, 947-958.
2. Coleman, C.E., Larkins, B.A. The prolamins of maize. In Seed Proteins; P.R. Shewry, R. Casey, Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1998, pp 109-139.

3. Wallace, J.C.; Lopes, M.A.; Paiva, E.; Larkins, B.A. New methods for extraction and quantitation of zeins reveal a high content of γ-zein in modified opaque-2 maize. Plant Physiol. **1990**, *92*, 191-196.

4. Wang, J.-F.; Geil, P.H.; Kolling, D.R.; Padua, G.W. Analysis of zein by matrix-assisted laser-desorption/ionization mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 5849-5854.

5. Adams, W.R.; Huang, S.; Kriz, A.L.; Luethy, M.H. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of zeins in mature maize kernels. *J. Agric. Food Chem.* **2004**, *52*, 1842-1849.

6. Woo, Y.-M.; Hu, D. W.-N.; Larkins, B.A.; Jung, R. Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. *Plant Cell.* **2001**, *13*, 2297-2317.

7. Drochioiu, G.; Ciobanu, C.I.; Bancila, S.; Ion, L.; Petre, B.A.; Andries, C.; Gradinaru, R.V., Murariu, M. Ultrasound-based protein determination in maize seeds. *Ultrason. Sonochem.* **2016**, *29*, 93-103.

8. Bancila, S.; Ciobanu, C. I.; Murariu, M.; Drochioiu, G. Ultrasound-assisted zein extraction and determination in some patented maize flours. *Rev. Roum. Chim.* **2016**, *61*, 725-731.