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Characterization of a 19 kDa α-Zein of High Purity

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A highly pure α-zein was extracted from corn flour using ethanol (95%). Subsequently, ion-exchange chromatography was performed, using SP-Sepharose that yielded a highly homogeneous protein. This protein migrated as a single band in 20% SDS−PAGE and in pH gradient gels, showing an isoelectric point of 6.8. Mass spectrometry (MALDI-TOF-MS) showed a single peak with a molecular mass of 24 535 Da. It was identified as Z19, when comparing the sequence obtained in an automatic Edman sequencer with the Swissprot database using BLAST. The molar extinction coefficient, determined by dry weight in 70% methanol, was 12 415.49 M^{-1} cm^{-1} at 280 nm. Light scattering showed its presence in a monodispersed state of 44−66 kDa aggregates in methanol (70%). Circular dichroism spectra allowed the estimation of an α-helix content that was lower than the one found for a mixture of two α-zeins but with a higher content of β sheets.

KEYWORDS: Zein; protein characterization; Z19; MALDI-TOF-MS; circular dichroism

INTRODUCTION

The structural analysis of hydrophobic proteins poses a challenge to traditional techniques because of their limited solubility in aqueous solvents (*1*). Such is the case of corn prolamins, also known as zeins, which are storage proteins that are the most abundant group in corn endosperm and are poor in lysine and tryptophan (*2, 3*). They are characterized by their high hydrophobicity and are classified as α, β, γ, or δ depending on their solubility, molecular weight, immunological response, and structure (*4*−*6*). Among these four groups, α-zeins are the most abundant and comprise 75−85% of total zeins; Z19 and Z22 proteins are included in this group and have apparent molecular weights of 23.8 and 26.7 kDa, respectively (*7*).

There are several reports on the separation of these proteins (*8*−*10*), but their close similarity has made separation and purification in a native state a difficult task, with low yields and poor quality of performance in further characterization studies. Therefore, the development of a suitable method to achieve a proper characterization remains an interesting challenge (*10*).

Zeins are more hydrophobic than prolamins from other cereals and tend to form aggregates and to precipitate under standard separation conditions used successfully to purify prolamins from wheat, rye, and rice (*1, 11*). Thus, most of the physicochemical and structural studies performed with these proteins have used the mixture of α-zeins (*12*−*18*). Furthermore, results produced with these mixtures have so far generated controversies about conformation (*13, 14*), which Forato, et al. (*17*) attribute to the use of mixtures and not to individual α-zeins in the published studies. This is why the individual purification and characterization of each of the α-zeins has become an important issue that will allow the study of behavior as defined mixtures or as individual proteins. Also, a high degree of sample homogeneity is required to perform structural studies.

The purpose of this work was to achieve such a preparation and to show, with different analysis criteria, the high purity of the obtained protein: single bands in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and isoelectric focusing (IFE), a single peak in matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and light scattering. This study differs from those cited above since this comprises the structural characterization of an individual, highly pure Z19 and thus allows comparison of its behavior when it is a member of the mixture with Z22.

MATERIALS AND METHODS

Biological material. Mature waxy yellow dent corn seeds (*Zea mays* L.) kindly donated by Arancia Corn Products, S. A. de C. V. (Tlalnepantla, Mexico), were ground in a disk mill (Weber BROS. & White, Metal Works, Inc., U.S.A.).

α-Zein Extraction from Grain. The extraction was performed according to Dickey et al. (*19*) with a slight modification. Lipids from flour were extracted with hexane, and carotenes and xanthophylls were extracted with a chloroform−methanol mixture (2:1). Defatted, decolored flour was mixed with 95% ethanol (5:1 solvent−flour (v/w)) using orbital agitation for 12 h at 25 °C (New Brunswick Scientific Model R76, Edison, NJ). It was then centrifuged at 12 000× *g* for 30 min at 4 °C and the supernatant was recovered.

Ion-Exchange Chromatography. A cationic exchange column was used (SP-Sepharose 2.6 cm × 11 cm Amersham Biotech, Uppsala, Sweden). The mobile phase was a citrate buffer, 0.02 M with 70%

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methanol, pH 3.5. The sample was solubilized in this phase to reach a final concentration of 0.5 mg mL^{-1}. It was then filtered with a 0.22 μM membrane (Millipore, Ireland) and injected into a high-pressure liquid chromatograph (AKTA prime, Amersham Pharmacia Biotech, Uppsala, Sweden). The elution buffer had 0.7 M NaCl, and a gradient from 0 to 1.2 mM NaCl was performed. A 0.5 mL min^{-1} flux was used, and elution was monitored at 280 nm through a UV detector. The collected peak was concentrated by ultrafiltration using YM10 membranes (Amicon, Millipore, U.S.A.).

SDS−PAGE. All protein preparations were dialyzed against deionized water and lyophilized. They were resuspended in equal volumes of deionized water and buffer (0.125 M Tris-Cl, 4% SDS, 20% Glycerol, 10% BME, 5 M Urea and bromphenol blue 0.01%, pH 6.8) and then submitted to heating at 100 °C for 10 min. SDS−PAGE was performed according to Laemmli (20). Gels were silver-stained (Amersham Biosciences, Uppsala, Sweden) for band visualization.

Amino Acid Sequencing and Identification. After SDS−PAGE, the protein was electroblotted to a polyvinylidenedifluoride membrane (Immobilon, Bio-Rad), by the Towbin method (*21*), for further sequencing. The *N*-terminal sequence was determined by automated Edman degradation (*22*) on a gas-phase protein sequencer (LF 3000, Beckman Instruments, Irvine, CA) equipped with an online high-performance liquid chromatography system (Beckman System Gold, Beckman Instruments, Irvine, CA). HPLC equipment included a diode array detector with settings at 268 and 293 nm for signal and reference, respectively. The HPLC column used was a Beckman Spherogel Micro PTH (2 mm × 150 mm) column; standard Beckman sequencing reagents were used for analysis.

Molar Absorption Coefficient Determination. This coefficient was determined for the purified Z19 protein by the dry weight method (*23*), which was slightly modified as follows: Protein solution in 70% methanol was dialyzed at 4 °C against 70% methanol for 48 h, through a 10 000 MW-cut Spectrapore membrane. Dilutions from 5 to 30 mg mL^{-1} of protein solution were performed, and absorbance at 280 nm was assayed in cells with a light path of 1 cm; 70% methanol was used as a blank. Two milliliters of each dilution were taken and dried at 90 °C for 48 h. The samples were weighed, and the molar protein concentrations were calculated. The molar extinction coefficient was the slope of the absorbance graph against protein concentration. The assay was performed in quadruplicate, and the standard deviation was no higher than 10% of the medium value.

Mass Spectrometry. For MS measurements, the purified zein was dissolved in 200 μL of a methanol−water mixture (70:30). Nanoelectrospray tandem mass spectra were obtained on an Esquire ion-trap mass spectrometer (Bruker-Flanzen Analytical, Gmbh, Germany) in the positive-ion mode, as described by Jensen et al. (*24*).

Isoelectric Point Calculation. A prepared IFE acrylamide gel (Amersham Pharmacia, Uppsala, Sweden) was used, with a 3−9 pH gradient. The lyophilized sample was solubilized in 5 M Urea; and 1 μg was used per lane. Bands were visualized using a silver-staining kit (Amersham Biosciences, Uppsala, Sweden).

Circular Dichroism (CD) Spectroscopy. The CD spectra were recorded on a Jasco J-715 spectopolarimeter, baseline and solvent corrected. Spectra were run at 25 °C. Concentrations used were in the 0.08−0.25 mg mL^{-1} range for the purified Z19 and 0.003−0.05 mg mL^{-1} for the α-zein mixture to avoid the aggregation effect. Three scanning acquisitions were accumulated and averaged to yield the final spectrum in both cases. The results are expressed as mean residue ellipticity, $[\theta]_{mrw}$, using a value of 109.867 g mol^{-1} for the molecular weight of a mean residue. To estimate the secondary structure content, the CD spectrum was analyzed with the SELCON3 deconvoluting program (*25*). The spectrum was also analyzed with the CLUSTER software (*26*).

Dynamic Light Scattering. Light scattering measurements were performed in a multi-angle light scattering instrument (Zetasizer nanoseries Malvern Instruments, U.K.). To make the analysis, the α-zein mixture and the purified Z19 were dissolved in 100 μL of a methanol−water mixture (70:30) and filtered by 0.22 μm membranes (Millipore, Ireland). The measurements were made at 25 °C. Three measurements were accumulated and averaged. The results were analyzed with the Zetasizer nanoseries software (2003).

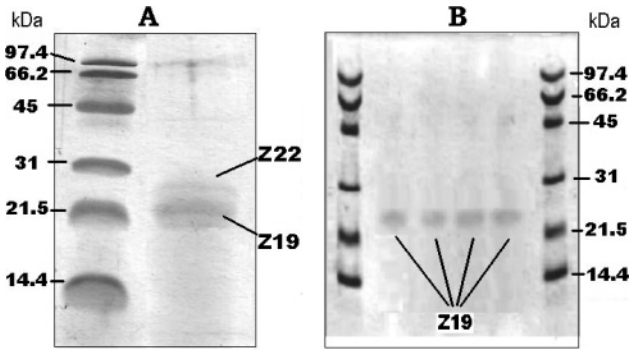

Figure 1. SDS–PAGE in acrylamide 20% of (A) the α-zein mixture after extraction with 95% ethanol and (B) the purified Z19 with 70% methanol and citric acid 0.02 N, pH 3.5.

RESULTS AND DISCUSSION

Purification and Identification of α-Zein. The mixture of α-zeins is observed as two typical bands of approximately 24 and 26 kDa, corresponding to Z19 and Z22 proteins, respectively, in SDS−PAGE (**Figure 1A**). The use of 95% aqueous ethanol allows the extraction of just the two α-zeins, avoiding solubilization of β, γ, and δ zeins, which yields an adequate work solution with an α-zein mixture concentration of 1.7 mg mL^{-1}. The final purification extraction yield was 2.63 ± 0.3 mg purified protein/100 g corn flour, which are remarkably good and enough for the next characterization experiments.

Various methods of protein purification were assayed according to several reports (results not shown). Wilson (*6*) and Paulis and Bietz (*9*) concluded that they had excellent zein separation using reversed-phase high-pressure liquid chromatography (RP-HPLC), but, according to our results, slight variations in equipment or accessories are enough to avoid having results such as the ones reported. Chromatographic profiles were different from those expected. Two peaks were obtained and were further analyzed by SDS−PAGE. Results showed the two α-zeins in similar amounts, which suggested the formation of different aggregates in both fractions.

Hydrophobic interaction chromatography (HIC) has not been reported as a tool for purifying this kind of proteins, but due to their highly hydrophobic properties, it seemed an interesting option. However, the evaluation of several matrixes and working conditions did not allow the separation of the mixed zeins. Instead, protein aggregation became an even greater phenomenon, possibly due to their hydrophobic surfaces that interact strongly with the support matrix.

Subsequently, ion exchange chromatography (IEX) was assayed. Solvent selection proved to be critical for this kind of chromatography because of its denaturing effects on the purified zeins. Aqueous buffers with nonpolar solvents led to just small amounts of pure, but denatured, Z19 being obtained. It must be said that Kruger and Bietz (*27*) reported that 22 kDa polypeptides present in cereals in high concentrations show a tendency to aggregate, mainly due to their hydrophobic nature. Thus, the protein yield they reported was not enough to allow complete characterization. It was demonstrated that a composition for the best solvent for zein is where the C/O (carbon:oxygen) is around 0.7−1.3 (*28*), which is necessary to keep zeins soluble, but does not guarantee that they will not denature. According to Lawton (*29*), 70% aqueous acetone allows the handling of 20% (w/v) zein solutions, but our results showed that this solvent produces a highly denatured protein.

Besides, Forato et al. (*30*) showed that alcohol solubilization does not affect conformation for the zein mixture, so they were

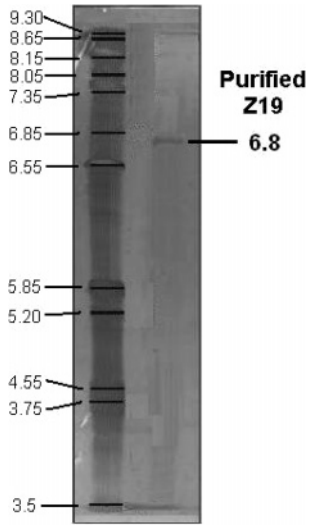

Figure 2. IFE of the purified Z19 protein. The pH gradient was 3–9, and the arrow shows the purified Z19 at pH 6.8.

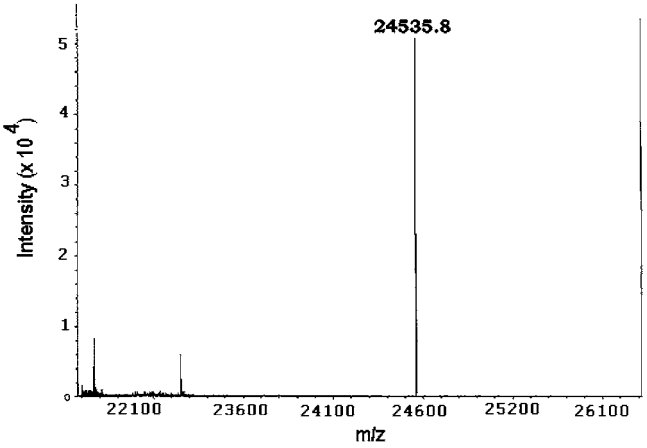

Figure 3. Purified Z19 profile by MALDI-TOF-MS.

able to validate secondary structure measurements in these solvents. Our results show that alcohols such as methanol, ethanol and 2-propanol, in concentrations ranging from 70 to 95%, make it possible to obtain native zeins in high concentrations in a nonaggregated state. Thus, zeins require careful solvent handling, as do materials and equipment, to avoid undesirable phenomena like aggregation and denaturation.

The purified protein obtained after IEX was eluted as a small fraction at 1.2 mM NaCl. **Figure 1B** shows a single band (19 kDa) corresponding to the purified protein.

IEF (**Figure 2**) made it possible to confirm that α-zein was the only protein present in the purified fraction and led to the determination that it has an isoelectric point of 6.8, just like the one reported for a zein mixture by Casella and Whitaker (*31*) and by Wilson (*32*).

A spectrum obtained for the purified protein using MALDI-TOF-MS is shown in **Figure 3**. A single peak appears at 24 535.8, a mass that can be assigned to one of the 19 kDa α-zeins (*15, 18*). No other peaks corresponding to molecular ions, double-charged peaks, or dimers could be seen, as in other studies, where two peaks of molecular ions have been reported for 19 kDa α-zeins (*15, 18*). The molecular weight for the present protein was determined using different methods. The molecular weights determined by SDS−PAGE and the analysis of the amino acid sequence (Predict Protein software) were 24 and 24.06 kDa, respectively, very close to that obtained

Table 1. Amino Acid Composition of the α-zein of 19 kDa Obtained from the Reported Sequence[a]

aa	Psch[a]	%	aa	Psch[a]	%
A	29	13.24	L	43	19.63
R	3	1.37	K	0	0.00
N	10	4.57	M	0	0.00
D	1	0.46	F	11	5.02
C	3	1.37	P	21	9.59
Q	43	19.63	S	18	8.22
E	1	0.46	T	7	3.20
G	2	0.91	W	0	0.00
H	1	0.46	Y	8	3.65
I	12	5.48	V	6	2.74

[a] Psch: amino acid residues per single polypeptide chain

experimentally using MALDI-TOF-MS, which was 24.535 kDa. The three methodologies used made it possible to obtain an average mass of 24.2 kDa for this Z19 protein. Comparison between the mass obtained by MALDI-TOF-MS and the one predicted for the corresponding amino acid sequence differs by 1.94%, which is remarkably good. Other authors (15, 18) reported molecular weights of 24.1 and 24.515 kDa from MALDI-TOF-MS for a Z19 and 24.706 kDa (18) from the amino acid sequence analysis. SDS−PAGE allows the classification of α-zeins into two groups (Z19 and Z22) according to the two bands obtained with this methodology, but techniques such as IFE (*29*), RP-HPLC (*1, 4−6*), or MALDI TOF-MS (*15, 18*) have shown that α-zeins consist of a mixture of at least 15 elements. Unlike other reports, in this study, just one of them was purified.

Sequencing and Identification of the Purified Zein. The sequence of the purified Z19 protein revealed that the first 20 amino acid residues were identified in a unique way, thus showing that the sample belonged to a homogeneous protein preparation. The *N*-terminus was TIFPQCSQAPIASLLPPYLP, which matches published sequences for zeins found in different data banks. The highest identity (100%) was obtained with the precursor of 19 kDa α-zein (100% identity) (Swissprot: accession number CAA26294). The analysis of the amino acid composition presented in **Table 1** shows that more than 50% of the amino acid residues are hydrophobic, with the highest levels corresponding to alanine, leucine, and proline, as with other α-zeins (*9, 34*). This structure yields high aliphatic indexes and high surface hydrophobicity (approximately 120 and 0.35, respectively) as calculated from the sequence analysis stored in the NCBI database using ProtScale software. The low amounts of polar, charged amino acids explain their high insolubility in water and the tendency to aggregate. The amount of aromatic residues is low, and there are no tryptophan residues.

The lack of tryptophan residues, together with the low tyrosine content, explains the low absorbance value at 280 nm and explains why the molar extinction coefficient obtained by the dry weight method (12 415.49 M^{-1} cm^{-1} ± 1350, r^2 = 0.9893) is higher than that obtained for proteins with a similar molecular weight but with a higher content of these residues (*35*). It is also higher than that predicted by Predict Protein software (10 240 M^{-1} cm^{-1}) or that performed according to the Hedelhoch method (*36*) (12 295 M^{-1} cm^{-1} in water at 280 nm). Another reason for obtaining such a high molecular coefficient would be the use of 70% methanol as solvent, which allows exposure of tyrosine residues and reduces insoluble aggregate formation, in contrast to the phenomena that occur when this protein is dissolved in water; furthermore, ϵ_{max} for tyrosine and phenylalanine increase when they are in alcoholic environments (*35*).

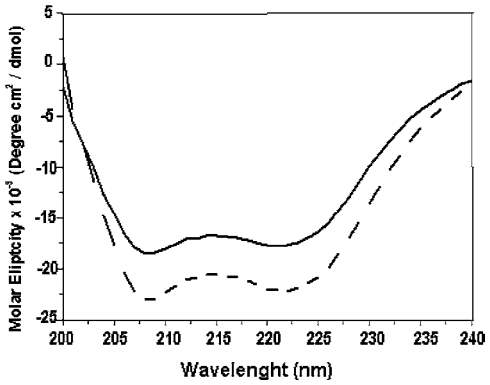

Figure 4. Far-UV CD Spectrum (*solid line*) for the pure Z19 protein an (*dashed line*) for the α- zein mixture in 70% (v/v) aqueous methanol at 25 °C

Table 2. Secondary Structure Content of a Zein Mixture and Purified Z19 as Determined by CD

	α-zein mixture (%)	purified Z19 (%)
α-helix	56.7	40.0
β-sheets	7.1	19.5
coils	8.2	15.4
not determined	28.0	25.1

Analysis of Secondary Structure by CD. CD spectra were determined both for the α-zein mixture and for the purified Z19. Aqueous methanol (70%) was used as solvent, and the 200−240 nm range was used (**Figure 4**). α-Helixes, β-sheets, and random coils were determined and are shown in **Table 2**.Corresponding spectra for both samples show two negative maxima around 207−208 nm and 222−224 nm that indicate high helical structure content. The highest percentage of α-helix was obtained for the two-zein mixture, with the same value as that reported in previous studies (*12*−*14*), while the β-sheet content was higher for the purified Z19. These results do not agree with those reported by Forato et al. (*17*) for a purified 19 kDa zein from a corn variety that does not produce Z22. They report an α-helix content of 46%, 22% of β-sheets, 23% of random coils and 13% of other structures. These differences might be due to the degree of homogeneity of the samples because the expression of Z22 in the modified corn used by Forato et al. (*17*) might be reduced but not totally blocked, while the expression of other zeins that are usually found in similar amounts could have been altered, resulting in a non-homogeneous mixture (*37*, *38*). The results obtained from the samples analyzed in this work correspond to the average of both the individual signals and those due to interactions among the mixture components. The prediction of a tertiary structure performed with the CLUSTER software indicated that the majority of the α-helixes in the Z19 folds into compact structures in both forms: purified and when it is found in the α-zeins mixture.

Light Scattering. Dynamic light dispersion is a useful diagnostic tool to obtain information about the aggregated state in proteins. The light-dispersion analysis made it possible to demonstrate that, under the conditions in which Z19 was obtained, it is found to be monodisperse in 2−3 monomer aggregates (**Figure 5**A). These forms seem to be very stable because they remain as such even under denaturing SDS−PAGE conditions (data not shown) with molecular weights between 44 and 66 kDa. This behavior is opposed to the one shown by the two zein mixtures (**Figure 5B**), which are found in larger sizes and molecular-weight aggregates, a fact that must explain

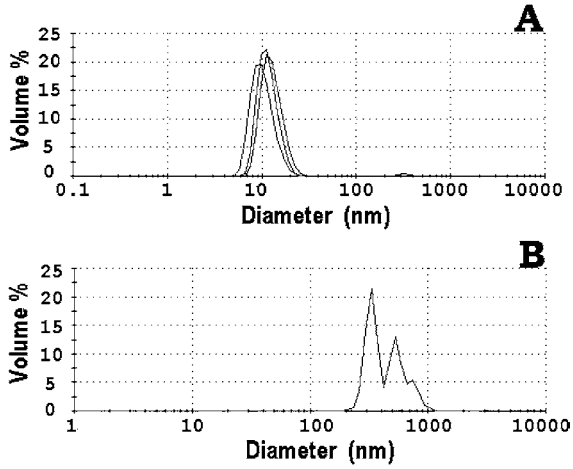

Figure 5. DLS profile, size distribution by volume for the (A) purified Z19 and the (B) α-zein mixture in 70% (v/v) aqueous methanol at 25 °C.

the differences in secondary structure content found by other authors.

The purification method reported here allows the acquisition of a highly pure, homogeneous preparation of a Z19 which provides the material required to understand its behavior, conformation, and structure, even though further studies are necessary. It is desirable to have the same quality of preparation for Z22 to allow a full understanding of the above features, both for the individual proteins and for the mixture, to be able to build a valid model of their structure and organization.

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