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Conformation of the Z19 Prolamin by FTIR, NMR, and SAXS

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The α zein, the maize storage prolamin, is a mixture of several homologous polypeptides that shows two bands in SDS-PAGE, called Z19 and Z22. The conformation studies carried out by several authors in this mixture are conflicting. To elucidate these inconsistencies, we analyzed the conformation of the Z19 fraction, extracted from BR451 maize variety by Fourier transform infrared spectroscopy, nuclear magnetic resonance, and small-angle X-ray scattering. The infrared results show that Z19 has 46% of α helix and 22% of β sheet. The fast N−H to N−D exchange measured by ^{1}H NMR spectroscopy showed that Z19 is not a compact structure. The scattering measurements indicated an extended structure with 12 by 130 Å. With these data, we have modeled the Z19 structure as a hairpin, composed of helical, sheet, turns, and secondary structures, folded back on itself.

KEYWORDS: Zein; prolamin conformation; Z19; protein structure; NMR; FTIR; SAXS

INTRODUCTION

The maize storage proteins are known as zeins and they have been studied because of their nutritional and technological applications (*1*, *2*). The zeins are an important protein source to human and livestock diet, because they represent 50% of the maize proteins (*2*, *3*). The zeins are the byproduct of starch and alcohol production and are used to protect nuts and medicines from humidity and oxygen in biodegradable materials (*1*, *2*).

The zeins are classified according to their solubility in α, β, γ, and δ zeins and are deposited as an insoluble mass in protein bodies (*4*). The α zeins are insoluble in water and are soluble in 70% ethanol. They represent more than 70% of the total prolamins present in maize, and they are characterized by two bands in the SDS/PAGE, with molecular rates from 23 to 24 KDa and from 26 to 27 KDa. These proteins are called Z19 and Z22, respectively. The cDNA and genes analysis indicated that these proteins have 210−220 and 240−245 amino acids and have homology (*2*, *4*−*6*).

The α zeins were among the first proteins studied by physical chemical methods. Since 1930, the structure of α zeins has been studied by ultracentrifugation, viscosimetry, birefringence and dielectric constant experiments. These studies, recently confirmed by Small angle X-ray scattering (SAXS) measurements (*5*, *7*), indicated that α zeins behave as an asymmetrical particle in solution. Secondary structure (SS) determination of α zeins by optical rotation (OR), optical rotatory dispersion (ORD), and circular dichroism (CD) (*5*−*7*), indicated high α helices content (40 to 60%) and 0−28% of β sheet. Using FTIR spectroscopy, we have recently demonstrated that the secondary structure of an α zeins mixture purified without solvent extraction has the same proportion of the ones extracted with alcoholic solution (46% of α helices and 28% of β sheet) (*8*).

There are no three-dimensional structures of α zeins determined by high-resolution techniques such as NMR and X-ray diffraction (*9*). The three-dimensional structure and packing of zeins and related proteins have been modeled by several authors (*7*−*10*). Argos et al. (*6*) and Garratt et al. (*10*), modeled α zeins as globular proteins, with 9 or 10 parallel helices of about 20 amino acids connected by loops. Both models were attractive because they allow a dense and regular packing of proteins inside the protein bodies but did not take into account the several experimental data which indicated that α zeins, in solution, have an extended conformation as well as a rodlike shape (*5*, *7*).

Tatham et al. (*5*) analyzed an α zeins mixture in methanol/water solution by SAXS and CD and by viscosimetry adding glycine to that solution (*5*). They concluded that these proteins behave as an extended molecule (14 × 150 Å for rod shape) in solution, and are mainly helicoidal, with some β sheet structures located in the N- and C-terminal regions, based on secondary structure predictions of a Z19 protein. The model suggested by Tatham et al. (*5*) were a continuous α helix structure or were with two α helix structures folded back with a loop or turn at middle. They justify the measurements in the α zeins mixture

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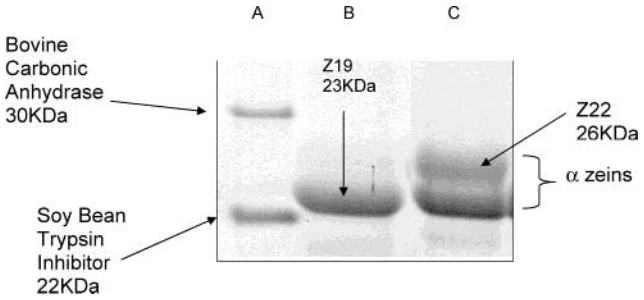

Figure 1. SDS/PAGE of standard molecular weights proteins, Z19, and α zeins. In well A are the standard proteins, carbonic anhydrase with 30 KDa and trypsin inhitor with 22 KDa. In well B, the Z19 proteins were applied and the typical band of this protein (molecular weight of 23 KDa) is shown, indicating its purity. In well C are the fractions that compose the α zeins as a mixture, Z19 with 23 KDa, and Z22 with 26 KDa.

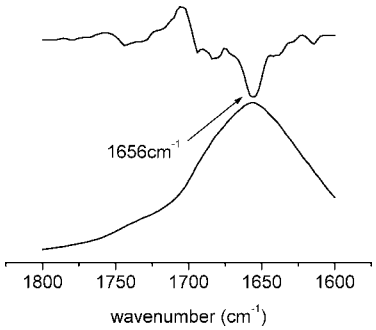

Figure 2. Infrared spectrum of Z19 protein at amide I region (1800–1600 cm^{-1}). Above the spectrum is shown its second derivative.

because there are sequence similarities between the Z19 and Z22 protein families.

Matsushima et al. (*7*) analyzed the α zeins structures by SAXS in ethanol aqueous solution with and without β-mercaptoethanol. They fitted their SAXS data using an elongated prism model (*7*). The model proposed uses the 12 Å of α helix diameter, 30 Å for the length of α helix repeats, similar to Argos et al. (*6*) and Garratt et al. (*10*) models but in a flat extended model giving the 130 Å dimension.

There is some controversy between Matsushima et al. (*7*) and Tatham et al. (*5*) in terms of experimental SAXS results. Matsushima et al. found a gyration radius 10% smaller than Tatham et al. Matsushima et al. attributed this difference to the fact that Tatham et al. analyzed the proteins without β-mercaptoethanol and also to the influence of the solvent in aggregation properties of a high hydrophobic molecule as zeins and others experimental differences (*7*).

This controversy cited above can also be due to the fact that both studies about the conformation of α zeins were made using the mixture of Z19 and Z22, which may vary according to genetic or environmental effects (*11*). To elucidate the inconsistencies found in zeins models, we analyzed the conformation of an isolated Z19 fraction from BR451 maize variety, which does not produce Z22 proteins (*12*), and analyzed it by NMR and SAXS in alcoholic solutions and also by FTIR in solid state. With these results, we proposed a structural model for the Z19 protein in solution.

MATERIALS AND METHODS

The Z19 protein was extracted by an alcoholic solution (70% ethanol) from de-fatted endosperm meal of BR451 maize variety, as described elsewhere (*13*). The purity of the protein was checked by SDS/PAGE (**Figure 1**).

SAXS Experimental Setup. The Z19 samples were dissolved in 90% ethanol and 10% water at a concentration of 3.6 mg/mL.

The SAXS experiments were carried out at Laboratório Nacional de Luz Síncroton (LNLS), Campinas, Brazil, using synchrotron radiation (SAS beamline). The wavelength of the incoming monochromatic X-ray beam was $\lambda = 1.743$ Å. A 1D X-ray position sensitive detector (PSD) was utilized to record the scattering intensity as a function of the modulus of the scattering vector $q = 4\pi/\lambda \sin \theta$, with θ being half the scattering angle. Parasitic scattering from air and beamline windows were subtracted from the total measured intensities. Desmearing of the experimental results was performed in order to correct for the effects of the rather large opening window of the 1D PSD (8 mm).

The sample-to-detector distance (1042.3 mm) was chosen to record the scattering intensity for q values ranging from 0.01 to 0.30 $Å^{-1}$. The parasitic scattering intensity produced by air and slits was subtracted from the total measured SAXS intensity. The samples were encapsulated inside a cell with two thin parallel mica windows.The resolution of the resulting solution X-ray scattering curve was extended to 21 Å.

The low resolution protein shape was restored using the ab initio procedure described by Svergun (*14*) as implemented in the program GASBOR. In this method, a dummy residue model (DRM) is generated by a random-walk C_α chain and is folded in a way to minimize a discrepancy between the calculated and experimental curves. The program simulates the protein internal structure, which makes it unnecessary to subtract a constant from the experimental data to ensure Porod's law (*15*). Several runs of ab initio shape determination with different starting conditions lead to consistent results as judged by from the structural similarity of the output models, yielding nearly identical scattering patterns and fitting statistics, in a stable and self-consistent process. The final shape restoration for the Z19 was performed using 217 dummy residues and 200 water molecules. The Z19 sequence was used to obtain the ab initio models. The Primary Acession Number in Swiss-Prot is Q946V7 (*16*).

Secondary Structure Prediction. A consensus of 10 Z19 protein sequences was obtained. The secondary structures were predicted by a consensus of six prediction secondary structure algorithms (GORIV, HNNC, Predator, SIMPA96 e SOPM) (*17*).

FTIR. The Fourier transform infrared (FTIR) spectra were recorded using a Perkin-Elmer Paragon 1000 spectrometer with 16 scans and 4 cm^{-1} of resolution, from 4000 to 400 cm^{-1}. The protein samples were lyophilized and prepared as KBr matrix (1 mg of protein and 100 mg of salt). To calculate the proportion of secondary structure of zeins, we used a calibration matrix obtained by singular value deconvolution (SVD) (*16*). The protein spectra were baseline corrected by subtracting a straight line from 2100 to 900 cm^{-1} and then normalized from 1800 to 1200 cm^{-1}. The amide I region from 1800 to 1600 cm^{-1} was multiplied by the calibration matrix to yield the proportion of secondary structure elements (α helices, β sheet, turns, and other structures) (*18*).

NMR. The NMR spectra were acquired using a Varian Inova 400 spectrometer. The ^1H solution spectra of Z19 were recorded in (70%) deuterated ethanol and 30% D_2O at 23 ± 1 °C, using 32 scans, a $\pi/2$ pulse, and a 3 s repetition time.

RESULTS

Figure 1 is the SDS/PAGE of the purified proteins. The single band of purified Z19 fraction (Mr 23 KDa) is shown in well B, and the two typical bands of α zeins at 23 KDa and 26 KDa corresponding to Z19 and Z22 proteins are in well C. **Figure 2** shows the FTIR spectrum and its 2nd derivative of Z19 sample from 1800 to 1600 cm^{-1}. In this figure, we can see that the amide I band is centered at 1656 cm^{-1}, and the 2nd has its strongest signal in the same position, indicating a high α helical content. The secondary structure of Z19 was quantified with a pattern recognition method (*18*), giving 46% of α helix, 22% of β sheet, 23% of turns, and 13% of other structures, similar to the ones obtained for the α zeins mixture (*18*).

The Guinier plots from the SAXS experiments indicated that the Z19 protein has a gyration radius (R_g) of 38 (±1) Å. **Figure 3** shows the pair distribution, $p(r)$, which is a measure of the

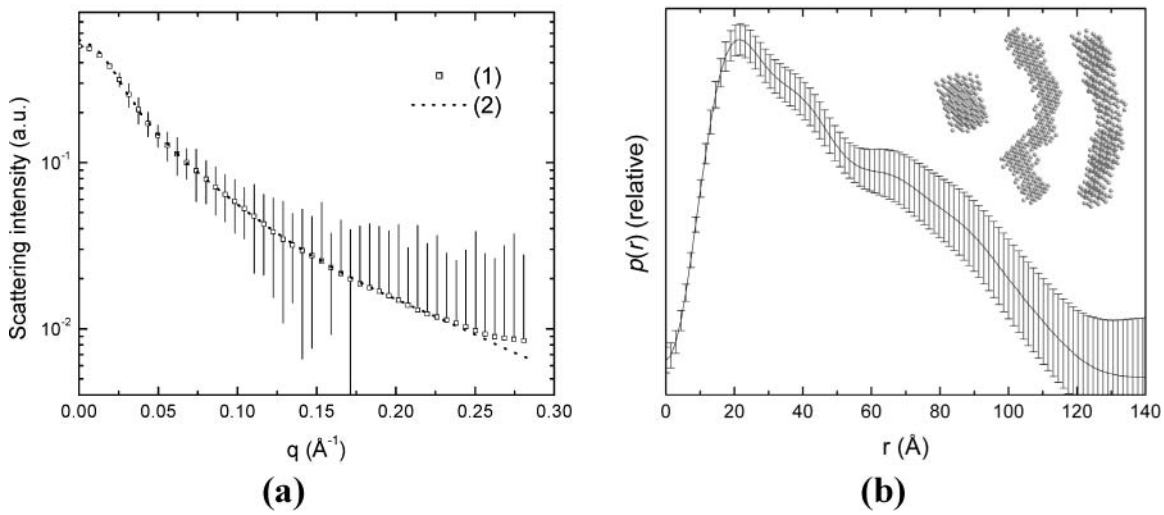

Figure 3. Experimental SAXS curves of Z19 protein in solution. (a) Plot of Log I versus *q* (logarithmic scale) (1) Experimental curve; (2) Scattering intensity computed from the DRM model (GASBOR). (b) Pair distance distribution, *p*(*r*), of Z19 showing the typical asymmetry that is expected for elongated (prolate) scattering objects. The maximum diameter, D_{max} = 120(10) Å (in the inset three different views of the restored shape are shown).

frequency of interatomic vector lengths within the proteins. This function is very sensitive to the overall asymmetry and domain structure within the particle. The shape of the *p*(*r*) function indicated that the Z19 protein is a very elongated prolate shape with a maximum dimension of 120 (±10) Å. A restored DRM of Z19 protein is also indicated in **Figure 3**.

The Z19 shape is illustrated in the inset of **Figure 3b**. To verify the uniqueness of the resulting shape, several independent restorations were performed using different starting conditions yielding similar results. The illustrated shape is a mean model composed of 10 independent ab initio models (three views from the same are shown). Unambiguous determination of the spatial positions of secondary structure elements is not possible, but an idea about the overall shape of the molecule could be obtained, as can be seen in the inset of **Figure 3b**. The overall dimensions of the Z19 shape indicate a protein with a maximum distance of around 120−130 Å and transverse dimensions ranging from 20 to 40 Å.

The N−H to N−D exchange in deuterated alcoholic solution measured by NMR was less than the few minutes necessary to sample preparation and analysis, indicating an extended or open structure.

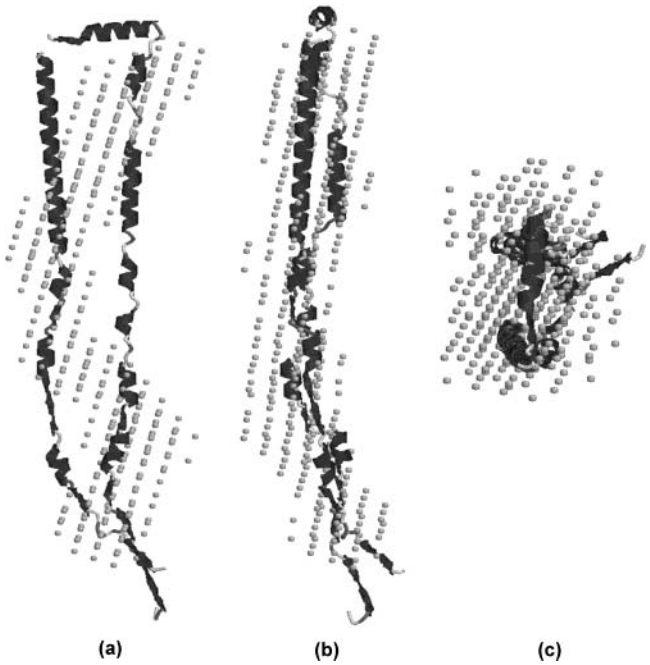

Figure 4. Proposed haipin model and Dummy residue model. The hairpin model for Z19 protein superposed to the *ab initio* DRM (the same as displayed in Figure 3b inset) (a) top view, (b) side view, and (c) same as in (b) rotated by 90°.

DISCUSSION

The FTIR spectrum in **Figure 2**, its 2nd derivative, and the secondary structure calculated by a pattern recognition method (*18*) indicated that Z19 has a high proportion of α helices (46%). This agrees with the α zeins measurements by ORD and CD in alcoholic solution (40−59%) (*6*, *7*) and by FTIR in KBr pellets (43%) (*8*). The amount of *β*-sheet structure measured by FTIR (22%) is higher than the ones measured for α zeins by CD in alcoholic solution, 0−20% (*6*, *7*), and lower than the ones measured by FTIR (28%) (*8*). The lower amount of *β* sheet calculated from FTIR spectrum may reflect the lower amount of this structure in Z19 than in Z22 polypeptides. The explanations for the higher *β*-sheet content measured by FTIR when compared to CD results in solution were already discussed elsewhere (*8*).

Another important insight that we can obtain from FTIR data is about α helix length. The Z19 does not have long helix segments of a continuous α helix or with a single turn or loop in the middle of two helical segments (*5*), because the amide I band is at 1656 cm^{-1}. The long α helix structures shift the amide I band to a lower wavenumber. The amount of shift depends on the helical dimension. For tropomyosin (with 40 amino acids long helix), the amide I absorption is in 1647 cm^{-1}, and for polylysine (extremely long helix) it is in 1637 cm^{-1} (*19*). These data are consistent with models proposed by Argos, Garratt, and Matshushima, which use helices of about 20 amino acids.

The SAXS measurements indicate a gyration radius of 38 Å (**Figure 3**) for Z19, smaller than the ones reported by Matsushima et al. and Tatham et al. for α zein mixture. This is expected because our protein is the smaller polypeptide fraction of the α zein mixture. Our measurements for pennisetin from Pearl millet, similar to Z22 fraction, gave a gyration radius of 41 Å, similar to α mixture (*20*). Using simulated curves for Z19 as extended or globular shapes, we found that the best

fittings are the more extended forms. However, our model differs from that of Matsushima et al., because the helical structures are arranged in a way that they are connected by other structuresas loops or sheets but not as parallel helices, as they proposed (*7*). The length of Z19 is more or less the same as the value obtained by Matsushima et al. for α zeins, (14 nm). The model in **Figure 4** also agrees with the high amount of short helix obtained by FTIR and the fast N−H to N−D exchange measured by NMR, suggesting that the hydrogen atoms are exposed to the solvent. The model is also in agreement with the SAXS data obtained for α zeins in the literature (*5*). The model that we are proposing also fits the dimensions proposed by Matsushima et al. (13- × 1.2- × 3-nm^3) in a more usual and stable conformation. Our hairpin model consists of short helical structures arranged in an extended way connected by loops, turns, or sheets (**Figure 4**). This model differs from the one proposed by Tatham et al., because our helices are in short segments (supported by FTIR data) and not with a single turn or loop in the middle of two long helical segments. We propose that the helices would fold back upon themselves or would be extended depending on the environment, instead of a unique α helix folded upon itself, as proposed by Tatham et al. Our model is also attractive because it is not very sensitive to hundreds of α zeins isoforms that can be expressed in maize endosperm. The model can also explain the ability to form fiber with α zeins. The extended structure has been proposed to other prolamin such as the gliadin and glutelins from wheat, secalin from rye, hordein from barley, and pennisetin from pearl millet. Extended structures are also known in single amino acid polypeptides or with repetitive sequences as present in zeins (*21*).

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