ABSTRACT:
The proline-rich N-terminal domain of γ-zein has been reported in relevant processes, which include its ability to cross the cell membranes. Evidences indicate that synthetic hexapeptide (PPPVHL), naturally found in N-terminal portion of γ-zein, can adopt the polyproline II (PPII) conformation in aqueous solution. The secondary structure of γ-zein in maize protein bodies had been analyzed by solid state Fourier transform infrared and nuclear magnetic resonance spectroscopies. However, it was not possible to measure PPII content in physiological environment since the β-sheet and PPII signals overlap in both solid state techniques. Here, the secondary structure of γ-zein has been analyzed by circular dichroism in SDS aqueous solution with and without dithiothreitol (DTT), and in 60% of 2-propanol and water with DTT. The results show that γ-zein has high helical content in all solutions. The PPII conformation was present at about 7% only in water/DTT solution. © 2007 Wiley Periodicals, Inc. Biopolymers 89: 175–178, 2008.

Keywords: γ-zein; maize prolamin; protein secondary structure; circular dichroism; polyproline II helix

INTRODUCTION

γ-Zein, a maize storage protein (maize prolamin) is composed of three protein fractions—Z16, Z27, and Z501,2—which are soluble in water only under reducing condition. Although γ-zein represents only 5–10% of the total zeins in normal maize, it has an important role in the formation and stabilization of protein bodies (PB)—accretions in the rough endoplasmic reticulum of maize endosperm cells where zeins are deposited. The proline-rich repetitive domain of the hexapeptide (PPPVHL)8 in the N-terminal of Z27, representing about 20% of the amino acids, has been suggested as responsible for the aggregation of γ-zein at the PB surface.3,4 In contrast to γ-zein, the synthetic hexapeptide (PPPVHL)8 is readily solubilized in water and adopts the polyproline II (PPII) conformation, an extended left-handed amphipatic helix, which has been characterized by Circular Dichroism (CD).5,6
Amphipathicity is one of the essential characteristics for a peptide to be able to cross a cell membrane. Consequently, the Z27 proline-rich hexapeptide (PPPVHVL) has been related to a new family of cell-penetrating peptides, which have been considered as potential vectors to carry drugs that have low bioavailability across cell membranes.\(^7\)\(^,\)\(^8\) As \(\gamma\)-zein is probably involved in such relevant processes, the conformational studies are very important to help understand its function.

The conformation of \(\gamma\)-zein extracted from a mixture of \(\beta\) and \(\gamma\) zein, was analyzed in aqueous solution under reducing conditions, by CD and optical rotatory dispersion and shows 26% \(\alpha\)-helix, 24% \(\beta\)-sheet, and 49% unordered structures.\(^9\) Recently, the secondary structures of \(\gamma\)-zein were analyzed directly in PB, the physiological state, by Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopies.\(^10\) The analyses were performed in the lyophilized PB purified from the maize cultivars BR473 and Poza Rica, and showed similar proportions of \(\alpha\)-helix and \(\beta\)-sheet, 33% and 31%, respectively.\(^10\) It was not possible to measure PPII content in \(\gamma\) zein, as proposed for the synthetic hexapeptide (PPPVHVL) in solution,\(^3,\)^\(^6\) since the signals for \(\beta\)-sheet and PPII conformations overlap in both solid state FTIR and NMR spectra.

Here, the secondary structure of \(\gamma\)-zein has been analyzed by CD in SDS aqueous solution with and without dithiothreitol (DTT), and in 60% of 2-propanol and in water with DTT. The results show that \(\gamma\)-zein has high helical content in all solutions. The PPPII conformation was present in about 7% in water/DTT solution.

**MATERIALS AND METHODS**

**Protein Bodies Purification and Characterization**
The PB rich in \(\gamma\)-zein were purified from BR473 maize variety by simple centrifugation, without using sucrose gradients.\(^11\)\(^,\)^\(^12\) Maize grains, collected 21 days after pollination, were ground in a blender with buffer A [Tris-HCl 200 mmol L\(^{-1}\); sucrose 200 mmol L\(^{-1}\); KCl 60 mmol L\(^{-1}\); MgCl\(_2\) 50 mmol L\(^{-1}\); pH 8.5] and centrifuged at 500 g for 20 min. The supernatant was centrifuged at 40,000 g for 1.5 h. The residue from this last centrifugation was washed with water, frozen, and lyophilized.

The lyophilized samples were solubilized in 0.1 mol L\(^{-1}\) SDS aqueous solution, with and without 1% of 2-mercaptoethanol (2-ME) as a reducing agent, in water and in 60% 2-propanol/water solutions with 2-ME. These samples were analyzed by 15% Comassie-blue-stained sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE). For UV-visible absorption analysis the sample was solubilized in 0.01 mol L\(^{-1}\) SDS aqueous solution without using reducing agent. The UV-vis spectrum was acquired from 200 to 200 nm and the sample concentration was approximately 0.2 mg mL\(^{-1}\).

**Liquid Chromatograph**
For liquid chromatography analysis (LC), the BR473 PB sample was prepared at 10 mg mL\(^{-1}\) of concentration, in 55% 2-propanol, 40% water, and 5% trifluoroacetic acid. The LC analysis was performed in Shimadzu chromatograph, with SPD-M10A Diode Array Detector, CBM-10A Communications Bus Module, and LC-10AD pumps A and B. A C\(_{18}\) analytical column (Bio-Rad, 4 mm ID × 250 mm length, 5 µm particle size) was used. The mobile phase used was (A) water/0.08% trifluoroacetic acid and (B) acetonitrile/0.08% trifluoroacetic acid. The injection volume was 5 µL. Sample components were separated at 0.8 mL min\(^{-1}\) with a linear gradient of mobile phase (B) initiated from 20% (v/v) to 100% (v/v) in 85 min. The analytical column effluent was monitored at 214 nm.

**CD Analysis**
CD measurements were performed with a JASCO J715 spectropolarimeter. Spectra were acquired from 250 to 190 nm, with 16 acquisitions, 1 nm bandwidth, 0.2 nm of resolution, and 1 mm path-length cells. The 0.2 mg mL\(^{-1}\) protein samples were prepared in 0.01 mol L\(^{-1}\) SDS aqueous solution without reducing agent. The samples were also dissolved under reducing condition, with 5 mmol L\(^{-1}\) of DTT, in 0.01 mol L\(^{-1}\) SDS aqueous solution, 60% 2-propanol aqueous solution and in water.

Secondary structures were determined by SELCON3, CONTIN, and CDSSSTR programs, using a protein data base (IBase = 5) which indicate \(\alpha\)-helix, \(\beta\)-sheet, turns, PPPII, and unordered structures proportions.\(^13\)\(^-\)\(^16\) From these spectra the tertiary structure class was also obtained by cluster analysis,\(^17\)\(^-\)\(^19\)

**RESULTS AND DISCUSSION**

**Solubilization and Characterization of \(\gamma\)-Zein**
Figure 1A shows the SDS/PAGE of BR473 PB extract in SDS aqueous solution. The bands at about 30 kDa and 50 kDa were attributed to the 27 kDa \(\gamma\)-zein (Z27), expressed in high concentration in this cultivar, and to the Z50 \(\gamma\)-zein, respectively.\(^10\) The Z16 \(\gamma\)-zein band was very weak, indicating its very low content in these PB. The band at about 42 kDa has been observed in zeins electrophoresis patterns,\(^7\)\(^,\)\(^10\) although it has not been identified yet.

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**FIGURE 1** SDS/PAGE electrophoresis (15%) stained with Comassie blue of \(\gamma\)-zeins from BR473 (lane 2), solubilized in (A) SDS and 1% 2-ME, (B) 60% 2-ProOH and 1% 2-ME solution, (C) water solution containing 1% 2-ME. Lane 1 contains the molecular masses markers.

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Figures 1B and 1C show the SDS/PAGE of the proteins extracted from BR473 PB by 60% 2-propanol solution and water, respectively, both containing 1% 2-ME. Figure 1B shows a strong band for Z27 and weaker bands for Z16 and Z50 zeins. The gel did not show the 42 kDa band indicating that only $\gamma$-zein were solubilized in this solution. The SDS/PAGE pattern of the sample solubilized in water with reducing agent was similar to that one in Figure 1A.

The BR473 PB content was also analyzed by LC, which has been considered an efficient method for separation and characterization of zeins groups. The chromatogram (Figure 2) shows a very intense peak, with elution time at about 35 min, attributed to $\gamma$-zeins. Dombrink-Kurtzman analyzed the zeins composition from several opaque mutant maize varieties that has a zein composition similar to BR473 and observed one intense peak with elution time around 28 min, corresponding to $\gamma$-zein, and others less intense peaks from 38 to 60 min, attributed to $\alpha$-zein. Differently from Dombrink-Kurtzman, the chromatogram of Figure 2 did not show two peaks for $\gamma$-zein, suggesting a unique or a predominant protein fraction. Paulis and Bietz have also analyzed zein extracts from mutants and normal maize and observed that $\gamma$-zein was eluted when the acetonitrile concentration was around 52%. In our chromatogram the acetonitrile concentration was about 54% for the most intense peak. Such similarity between these results suggests that the protein component, responsible for the peak in both cases, must interact with the column in a similar way. Since the column used by Paulis and Bietz was the same ($C_{18}$), we can conclude that the hydrophobic characteristics of proteins present in BR473 sample are similar, indicating the same $\gamma$-zein.

**Conformational Analysis by CD**

Figure 3A shows CD spectra of the PB proteins in SDS aqueous solution with and without DTT. The spectra are quite similar and characterized by two negative broad peaks at about 208 and 220 nm and a positive one around 191 nm, typical of proteins with $\alpha + \beta$ structure, in which the $\alpha$-helix band intensity is stronger than the $\beta$-sheet structure.

**FIGURE 2** LC chromatogram of $\gamma$-zein sample. Mobile phase: A, H$_2$O + 0.1% TFA; B, ACN + 0.1% TFA. Gradient: 0.01 min, 20% B; 85 min, 100% B; 90 min, 100% B; 95 min, 20% B. Solvent flow: 0.8 mL min$^{-1}$. Column: C$_{18}$, 250 mm $\times$ 4.0 mm (Bio-Rad). UV detection: $\lambda$ = 214 nm.

**FIGURE 3** CD spectra of $\gamma$-zeins from BR473 acquired from 250 to 190 nm. (A) SDS (---) and SDS containing DTT (----); (B) SDS/DTT, 60% 2-propanol/DTT, and water/DTT.
Table 1  Secondary Structure Proportions Obtained from Deconvoluted CD Spectra of γ-Zeins in SDS/DTT, 60% 2-Propanol/DTT, and Water/DTT

<table>
<thead>
<tr>
<th>Solution</th>
<th>α-Helix (%)</th>
<th>B-Sheet (%)</th>
<th>Turns (%)</th>
<th>PPII (%)</th>
<th>Unordered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrOH/DTT</td>
<td>34 ± 1.0</td>
<td>6 ± 1.0</td>
<td>1 ± 0.5</td>
<td>7 ± 0.5</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td>SDS/DTT</td>
<td>2 ± 0.1</td>
<td>6 ± 1.0</td>
<td>1 ± 0.5</td>
<td>7 ± 0.5</td>
<td>7 ± 0.5</td>
</tr>
<tr>
<td>H2O/DTT</td>
<td>55 ± 4.0</td>
<td>6 ± 3.5</td>
<td>10 ± 0.6</td>
<td>7 ± 1.5</td>
<td>24 ± 5.5</td>
</tr>
</tbody>
</table>

* RMSD, root mean square deviation.

The γ-zein conformation in 60% 2-propanol and water solutions, both containing DTT, was also analyzed by CD (Figure 3B). The spectrum in propanol shows small differences in peak intensities and wavelengths when compared with the one obtained in SDS solution with DTT. This indicates small protein conformation changes in these two environments. In contrast, the spectrum of γ-zein in water/DTT is very different from the others (Figure 3B). It shows a shift in the negative maximum from 208 to 206 nm and a reduction in the intensities at 220 and 191 nm. This indicates a major conformation change caused mainly by the solvent, since the reducing agent was used in all solutions. The spectrum of γ-zein in water is not so similar to the ones observed to proteins with PPII conformation.5,6,24 It is consistent with a protein containing approximately 50% α-helix and unordered PPII conformation.

The γ-zein secondary structures content for all reducing preparations were calculated from CD spectra using deconvolution programs, SELCON3, CDSSTR, and CONTIN, and the protein data base, identified as IBase 5.16 The averaged results of the three methods and the standard error are in Table I. The results show very high helical content for SDS and 2-ProOH solutions, 87% and 85%, respectively. The others secondary structures in these solutions were only about 15%. On the other hand, in aqueous solution, the α-helix content was 55% with a three-fold increase in other secondary structure content. Thus, γ-zein shows higher sensitivity to solvent effect than α-zein, which shows similar secondary structures in the PB and in ethanol aqueous solution.25 The high α helical content in alcohol and SDS solutions should be due to the helix inducing effect of these solvents.

The PPII conformation was only 1% in SDS and about 4% and 7% in propanol and water solutions (Table 1). These results suggest the presence of PPII in the hexapeptide repetitive domain in native γ-zein, although the PPII content observed in aqueous solution is about 30% of PPII conformation predicted for the hexapeptide (PPPVHVL)8 in γ-zein, about 22%.10 In this protein, the PPII conformation seems to be partially suppressed even in water, as Val and Leu residues of the repeats are inimical to this conformation. This structure as observed in synthetic hexapeptide could be able to cross cell membranes and be involved in the formation and stabilization of PB. It has also been suggested that this domain is responsible for the aggregation of γ-zein at the PB surface.3,4

REFERENCES

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