Effect of Solvent and Temperature on Secondary and Tertiary Structure of Zein by Circular Dichroism

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ABSTRACT

Circular dichroism studies were performed on zein to determine how the secondary and tertiary structure changes with different solvents, temperatures, or pH. Alcoholic solvent type and common denaturants such as SDS and low amounts of urea had little effect on the secondary structure of zein. Utilization of dimethylformamide or acetic acid as solvent gave changes in tertiary structure. Solutions of zein in 8*M* urea produced solutions with large changes in tertiary structure. The dissolution of zein in 50 m*M* sodium hydroxide produces a zein with large changes in secondary and tertiary structure and little loss in primary structure. Increasing the temperature of zein to 70°C in 80% ethanol-water gave reversible changes in the primary structure (20% reduction in absolute magnitude of $[\theta]_\lambda$ at 208 and 222 nm) and tertiary structure (40% reduction in absolute magnitude of $[\theta]_\lambda$ at 268 nm).

Zein, the major storage protein of corn, is a prolamin byproduct of the corn milling industry. It was used extensively in the fiber and coating industries through the early 1960's. Manufacturers lost interest when cheaper petroleum-based products became available (Shukla et al 2001; Lawton 2002). There has been a renewed interest in using zein as well as other renewable materials in consumer goods because of concerns about the impact that petroleum-based products have on the environment (Lawton 2002). Today, zein applications are limited to food coatings and drug tableting (Shukla 1992). The development of new zein-based products that have broader applications is needed to improve the economics of the growing bioethanol industry. Historical applications of zein required modification using formaldehyde as a cross-linking agent (Yelland 1951; Uy 1998). If used today, this manufacturing technology would require significant investment to be performed safely. Other derivitization techniques will be required to provide a safe and economical process. Changes in protein secondary or tertiary structure can alter protein reactivity with chemical reagents (Rawel et al 2005). If protein secondary and tertiary structure could be modified to provide increased accessibility to the reactive sites of zein, further derivitization could be more readily accomplished. Solvent selection, pH, and temperature can alter protein secondary or tertiary structure (Chiou and Ueda 1994; Lin et al 1995; Berne et al 2005; Bhattacharjee et al 2005). Relative to other proteins, zein is unusual in that it is readily soluble in various organic solvents where the most common solvent system is water and ethanol at 60–95% (Lawton 2002). As the concentrations of ethanol are changed, significant changes can take place in protein secondary (Lin et al 1995) and tertiary structures (Chiou and Ueda 1994).

Another potential barrier that may limit the penetration of zein into other commercial applications is lot-to-lot variability. The subtle differences in zein produced by the same process have been shown to give solutions with significantly different rheology (Selling et al 2005). It was shown that a part of this difference could be attributed to differences in molecular weight. It was not determined whether there were structural differences within the zein samples. Differences in secondary structures may cause differences in tertiary structures (Voet and Voet 2004a). These tertiary structure differences will alter the protein radius of gyration, which may then affect properties such as static or dynamic viscosity (Allcock et al 1981; Cho et al 2006; Mohammadifar et al 2006). An improved understanding of the cause for the lot-to-lot rheological differences previously observed would be beneficial to any commercial operation where rheological variations can lead to irregular flow, pressure drop variation, and defects in the final article.

Far-UV circular dichroism (CD) has been used to characterize the structure of zein and to support structural models for zein (Danzer et al 1975; Argos et al 1982; Tatham et al 1993; Bugs et al 2004; Yong et al 2004; Cabra et al 2005). These studies have been in aqueous ethanol (EtOH) or aqueous methanol (MeOH), the most common solvents for zein, as other solvents such as N,N-dimethylformamide (DMF) cannot be used in this technique due to solvent interference. A far-UV CD study was recently conducted on laboratory-generated zein in 70% EtOH and water where the pH and temperature were varied (Cabra et al 2006). The average α-helical content in these studies was 50%, with the majority of the remaining structure in a random coil state. Altering protein secondary (Kosky et al 1999) and tertiary (Kossiakoff, 1988) structure can alter reactivity. A change in the secondary or tertiary structure of zein may improve access to certain reactive sites and make derivitization easier. CD using near-UV light (260–350 nm) has been used to study the tertiary structure of proteins (Bhattacharjya and Balaram 1997; Sridevi et al 2000; Ahmad et al 2005; Berne et al 2005; Bhattacharjee et al 2005; Kelly et al 2005). The absorbancies observed in this region result from aromatic amino acids of the protein. While direct structural information cannot be readily obtained from spectra using this technique, these spectra are useful in defining changes in a given protein structure due to changes in temperature, pH, or solvent. To date, there have been no CD studies conducted on zein using near-UV light.

Developing new uses for zein will require chemical modification (Biswas et al 2005). Understanding what conditions are necessary to change protein structure will help guide researchers in their efforts to develop appropriate conditions to alter zein. While it is known that certain conditions such as high temperature will alter the secondary structure of zein (Cabra et al 2006), there have been no studies of the tertiary structure of zein by examining the near-UV CD spectra of zein. The tertiary structure may not necessarily mean that the secondary structure has been greatly altered (Donnet et al 2001). Developing an improved understanding of how the secondary and tertiary structure of zein changes with changes in environment would help to develop new chemistries to alter the structure and properties of zein-based articles.

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MATERIALS AND METHODS

Materials and Equipment

Five lots of zein were obtained from two commercial sources: zein lots from Freeman Industries (Tuckahoe, NY) 40002371 (FZ2371; protein 90.1%, fat 1.9%, ash 1.2%, water 3.3%); 4000 3111 (FZ3111; protein 83.1%, fat 7.1%, ash 1.2%, water 4.4%); 40003121 (FZ3121; protein 89.0%, fat 5.0%, ash 1.3%, water 3.4%); and 40004081 (FZ4081; protein 89.5%, fat 3.0%, ash 0.6%, water 4.4%); and zein lot 040315-1 from Showa Sangyo (Tokyo, Japan) (SZ; protein 91.8%, fat 0.4%, ash 0.7%, water 6.9%). All aqueous solutions were prepared on a weight-to-weight basis using ultrapure water produced using a Nanopure system (Barnstead International, Dubuque, IA). Tris was received from Fisher Chemicals (Fair Lawn, NJ). Nupage 4–12% Bis-Tris gels, X-Cell Surelock mini cell, Nupage LDS sample buffer, and Mark 12 MW standard (Invitrogen, Carlsbad, CA) were used for the SDS-PAGE experiments. Dithiothreitol (DTT) was used as received from Molecular Probes (Eugene, OR). SDS was used as received from Bio-Rad Laboratories (Hercules, CA). 4-Morpholineethanesulfonic (MES) acid monohydrate and tris(hydroxymethyl) aminomethane (Tris) were used as received from Acros Organics (Pittsburgh, PA). All other chemicals were reagent-grade and used as received (Sigma-Aldrich, St. Louis, MO). Dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA) had a 1,000 molecular weight cut-off and was rinsed in water for 1 hr before use. Freeze-drying was accomplished using a freeze-dry system (Labconco, Lyph-Lock 4.5, Kansas City, MO).

Circular Dichroism Measurements

Zein samples were dissolved in the desired solvent and passed through a 5-µm Whatman nylon syringe filter. At the concentrations tested, the zein samples dissolved completely. The solutions were stored at room temperature in microcentrifuge tubes until testing, usually <12 hr. There was no evidence of protein precipitation or gelation over the course of several days. Zein far-UV and near-UV CD spectra were collected on a circular dichroism spectrometer (model 215, Aviv Biomedical, Lakewood, NJ). Far-UV CD spectra were obtained at 260–200 nm with a 1-nm bandwidth and temperature controlled at 25 ± 1°C. The far-UV CD samples were tested at 0.5 mg of zein/mL in quartz cells with a path length of 0.1 cm. Each zein spectrum was collected three times and averaged for the final result (SD for mean residue ellipticity was 4×10^5 mdeg cm^2/dmol), while each solvent spectrum was collected 10× and averaged. Near-UV CD spectra were obtained at 300–260 nm with a 1-nm bandwidth and temperature controlled at 25 ± 1°C. The near-UV CD samples were tested at 40 mg of zein/mL (except for the DMF solution which was tested at 34 mg of zein/mL) in quartz cells with a path length of 0.1 cm. Each spectrum was collected at least 10× and averaged (SD for mean residue ellipticity was 1.1×10^3 mdeg cm^2/dmol), except for runs where 50 m*M* sodium hydroxide (NaOH) or DMF were used as solvent, where SD was 2.0×10^3 mdeg cm^2/dmol. The CD data is expressed in terms of mean residue ellipticity, $[\theta]_\lambda$, where the units are mdeg cm^2/dmol. It is calculated as $[\theta]_\lambda$ = MRW $\theta_\lambda/10dc$, where mean residue mass (MRW) is 110 g/mol; θ_λ is measured ellipticity at the specific wavelength in mdeg; c is the concentration of protein in grams (dry basis)/cm^3 and d is the path length in cm (Kelly and Price 2000).

Circular Dichroism Measurements—Temperature Effects

A solution of FZ3121 zein in 80% EtOH at 0.5 mg/mL was prepared and filtered through a 5-µm Whatman nylon syringe filter. The far-UV CD spectra were recorded in the manner detailed above at 25–70°C at 10°C increments (except for the final increment where the temperature was increased from 65 to 70°C). Additional CD spectra were then obtained as the sample was cooled from 70 to 25°C at 10°C increments (except for the initial increment where the temperature was decreased from 70 to 65°C). At each temperature, spectra were recorded after the sample had equilibrated at that temperature for 15 min.

A solution of FZ3121 zein in 80% EtOH at 40 mg/mL was prepared and filtered through a 5-µm Whatman nylon syringe filter. The near-UV CD spectra were recorded in the manner detailed above at temperatures of 25, 50, and 70°C. Additional CD spectra were then obtained as the sample was cooled to temperatures of 50 and 25°C. At each temperature, the spectra were recorded after the sample had equilibrated at that temperature for 15 min.

SDS-PAGE

DMF was added to the zein to give a 2% protein solution as control. For high pH experiments, zein was dissolved in 50 m*M*

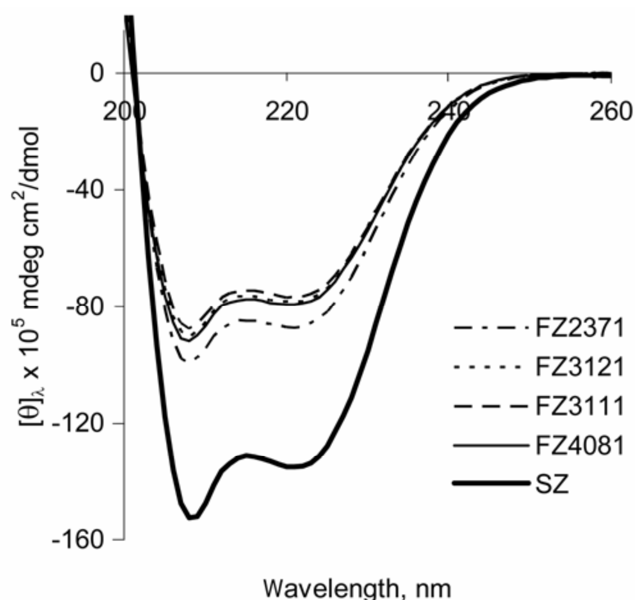

Fig. 1. Far-UV CD spectra of commercial zeins at 5 mg/mL in 80% EtOH at 25°C.

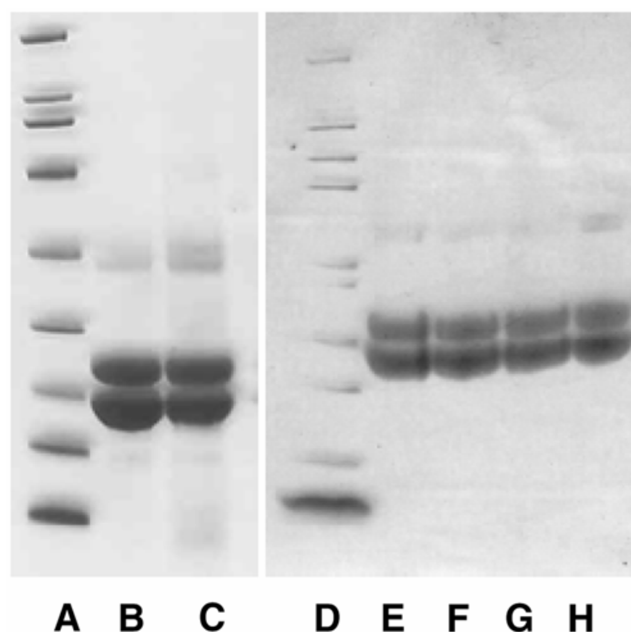

Fig. 2. SDS-PAGE gels. Lanes: **A,** molecular weight marker; **B,** FZ4081; **C,** SZ; **D,** molecular weight marker; **E,** FZ4081; **F,** FZ4081 50 m*M* NaOH 15 min; **G,** FZ4081 50 m*M* NaOH 30 min; **H,** FZ4081 50 m*M* NaOH 60 min.

NaOH, the solutions were stirred for 15, 30, or 60 min at room temperature; 10 μL of 50 m*M* DTT was added to 15 μL of the desired solution; 25 μL of Nupage LDS sample buffer and 50 μL of water. This solution was then placed in boiling water for 5 min. Into the wells, 10 μL of each sample was added and the mini cell filled with running buffer (9.76 g of MES, 6.0 g of Tris, 1.0 g of SDS, 0.3 g of EDTA-Na_2, in 1 L of water). Amperage was set to 100 mA by adjusting voltage. Gels were run until the dye front reached the desired location. Gels were then rinsed with ultrapure water and stained with Coomassie Brilliant Blue solution (1 g of Coomassie Brilliant Blue, 500 mL of methanol, 120 mL of acetic acid, 380 mL of water) for 1 hr. The gel was destained in solution (180 mL of EtOH, 80 mL of acetic acid, 740 mL of ultrapure water) overnight and then scanned.

RESULTS AND DISCUSSION

CD Spectra

An improved understanding of the secondary and tertiary structure of zein would be beneficial for defining lot variability for zein manufacturing processes and to determine how different processing conditions, presumably being used by different manufacturers, affect zein structure. The two negative peaks at 208 and 222 nm observed in the CD spectra indicate the presence of α-helix secondary structure and the intensity reflects the amount of α-helix present in the protein (Myer 1968; Chang et al 1978). Because there are many equations for estimating the amount of each type of secondary structural feature (Greenfield and Fasman 1969; Chen et al 1972; Hennessy and Johnson 1981; Provencher and Gloeckner 1981; Manavalan and Johnson 1987; Perczel et al 1991; Nohm et al 1992; Perczel et al 1992a,b; Andrade et al 1993; Sreerama and Woody 1993, 1994a,b, 2000, 2004; Sreerama et al 1999ab) rather than selecting a specific equation a priori, we will present mean residue ellipticity $[\theta]_\lambda$ and make broad conclusions regarding overall secondary structural features. The larger the absolute magnitudes of the negative peaks at 208 and 222 nm are indicative of the amount of α-helix present in the protein (Sreerama and Woody 2004). For the near-UV CD spectra, the 260–320 nm region contains absorbencies that result from the aromatic-containing amino acids. Tryptophan has absorbencies of 290–305 nm. Tyrosine has absorbencies of 275–280 nm. Phenylalanine has absorbencies of 255–275 nm. The magnitude of the absorbencies at each wavelength in this region will depend on the number of the aromatic-containing amino acids, their mobility, and their local environment (Kelly et al 2005). Taken alone, CD spectra measured in this region cannot readily contribute structural information. However, the technique can provide information about a change in the environment of the aromatic amino acid residues of a protein.

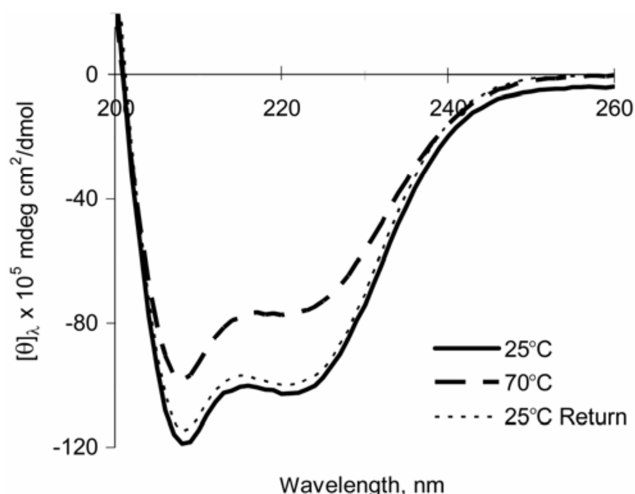

Fig. 3. Far-UV CD spectra of FZ3121 zein at 5 mg/mL in 80% EtOH at 25°C and 70°C.

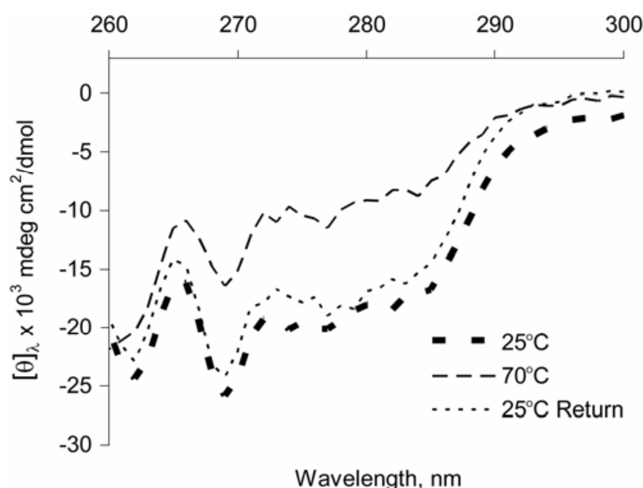

Fig. 4. Far-UV CD spectra of FZ3121 zein at 5 mg/mL in 60-90% EtOH at 25°C.

Previously, it was observed that different lots of zein from the same manufacturer had significantly different rheology (Selling et al 2005). To determine whether these differences may be, in part, related to differences in secondary structure, four different zein lots from Freeman and one zein lot from Showa had their far-UV CD spectra measured in 80% EtOH and water (Fig. 1). There is little difference between the lots of zein from the same manufacturer. Therefore the differences observed in rheology between these zein samples cannot be attributed to large differences in secondary structure. The Showa zein had more α-helical character than the Freeman zeins (the absolute magnitude of the $[\theta]_\lambda$ at 208 and 222 nm are >60% larger for the Showa zein). While SDS-PAGE composition of Showa zein was fairly similar to those supplied by Freeman (Fig. 2), it may have slightly lower MW protein because of the base present in its manufacture (Takahashi and Yanai 1996). SDS-PAGE is not the best choice for quantitative analysis, however it is clear that there are no gross differences between the zeins from these manufacturers. When CD spectra of FZ3121 and SZ zein were collected in the near-UV region, there were no significant differences observed (data not shown), indicating that there are no large differences in tertiary structure. The Showa zein may be produced by a different method and have higher purity than the Freeman zein (98.7% protein, dwb, for SZ vs. 93.1% protein, dwb for FZ3121). These results show that near-UV CD is a useful tool for defining differences in zein produced by different manufacturing methods.

TABLE I
Mean Residue Ellipticity (mdeg cm^2/dmol × 10^5) at 208 and 222 nm for an FZ3121 Solution at Various Temperatures[a]

Temperature (°C)	208 nm	222 nm
25	–119	–102
35	–112	–93
45	–106	–87
55	–103	–82
65	–99	–78
70	–98	–76
65	–101	–80
55	–106	–85
45	–110	–92
35	–113	–96
25	–115	–100

[a] FZ3121 at a concentration of 0.5 mg/mL in 80% EtOH/water (SD 2.0 × 10^5 mdeg cm^2/dmol).

CD Spectra—Temperature, Solvent, pH, and Denaturant Effects

Temperature and solvent type were altered to change the secondary or tertiary structure of zein and therefore potentially alter the accessibility of the reactive sites of zein. Denaturants that alter the structure of proteins (Creighton 1993) were added to zein solutions to determine how these reagents affect secondary structure (Voet and Voet 2004b). After each of these treatments, circular dichroism was used to monitor structural changes.

Processing temperature is an important variable for many proteins because proteins denature at high temperatures (Voet and Voet 2004b). With denaturation, irreversible structural changes can take place. Temperature is a very important process variable for most chemical reactions. When a solution of FZ3121 in 80% EtOH was heated from 25 to 70°C secondary structure changes took place as evidenced by changes in the far-UV CD spectra. A reduction in the absolute absorbance of $[\theta]_\lambda$ by >20% of the negative peaks at 222 and 208 nm was observed, which shows that α-helix character was lost (Fig. 3). The secondary structure changes that take place with heating are predominantly reversible upon cooling. Table I details the mean residue ellipticities at 208 and 222 nm for the FZ3121 solution in the heating and cooling cycles. The values for mean residue ellipticity are very close at each temperature. When SZ zein was heated to 70°C in 80% EtOH and water, it too lost α-helix structure. Absolute $[\theta]_\lambda$ at 208 and 222 nm were reduced by 34% (data not shown). When a solution of FZ3121 in 80% EtOH was heated from 25 to 70°C, tertiary structure changes took place as evidenced by changes in the near-UV CD spectra. As seen in Fig. 4, there is a change in the CD spectra with a 40% reduction in the absolute magnitude of $[\theta]_\lambda$ at 268 nm on heating, indicating that the local environment of the aromatic amino acids residues has changed, which would result from a change in tertiary structure. These tertiary structural changes are reversible; the spectra taken on returning to 25°C is comparable to the initial 25°C spectra. The near-UV CD spectra taken at 50°C during both the heating and cooling cycles lay in-between that of the 25 and 70°C spectra (data not shown). These experiments illustrate that reversible secondary and tertiary structural changes take place with heating.

To determine whether the fraction of EtOH in the aqueous solvent has an impact on the secondary structure of zein, a series of samples was tested where the % EtOH in water was varied from 60 to 90%. Within this series, little difference in secondary structure was observed (Fig. 5). Solvents other than aqueous EtOH may also have an effect on the secondary structure of zein. Aqueous isopropyl alcohol (IPA) and MeOH are known as good solvents for zein (Lawton 2002). Figure 6 shows that zein in 70% IPA undergoes larger changes than in the corresponding percentage of EtOH, with a 43% difference in the absolute magnitudes of the prominent negative peaks. The secondary structure of zein in 70% MeOH is not different (a 10% increase in the absolute magnitude of $[\theta]_\lambda$ at 222 nm) than zein in the corresponding EtOH solution. MeOH and EtOH are more polar than IPA (Wohlfarth 1999) and they are better solvents than IPA (Lawton 2002). As such, they may be better solvents for the zein, giving more similar structures in solution relative to IPA.

High pH water is an effective solvent for dissolving zein. When zein is dissolved in 50 m*M* NaOH (pH 12.7), there is a large change in secondary structure with 60–90% reductions in absolute magnitude of $[\theta]_\lambda$ at 208–222 nm (Fig. 6). The far-UV CD spectrum is indicative of a mostly random coil structure in this solvent. When the near-UV CD spectrum of zein was obtained in this solvent, large differences were observed relative to the 80% EtOH and water solution, indicative of changes in tertiary structure, a >90% reduction in absolute magnitude of $[\theta]_\lambda$ at 268 nm (Fig. 7) (Voet and Voet 2004a). Initially, it is not apparent whether these spectral changes are due to structural changes or protein degrada-

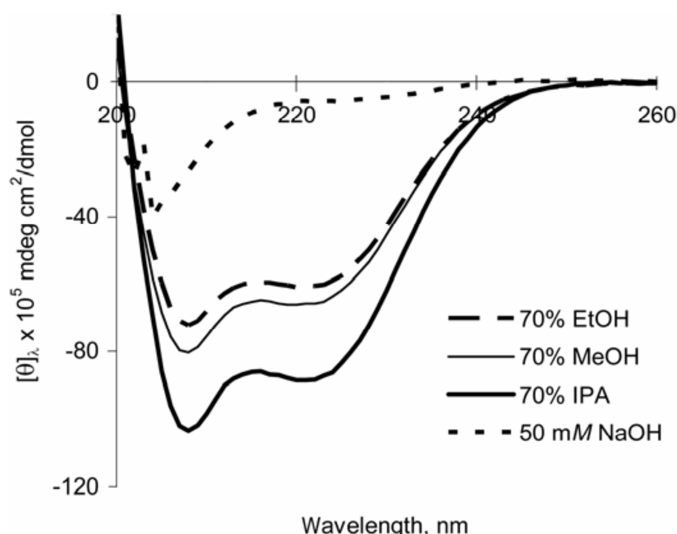

Fig. 6. Far-UV CD spectra of FZ3121 at 5 mg/mL in various solvents at 25°C.

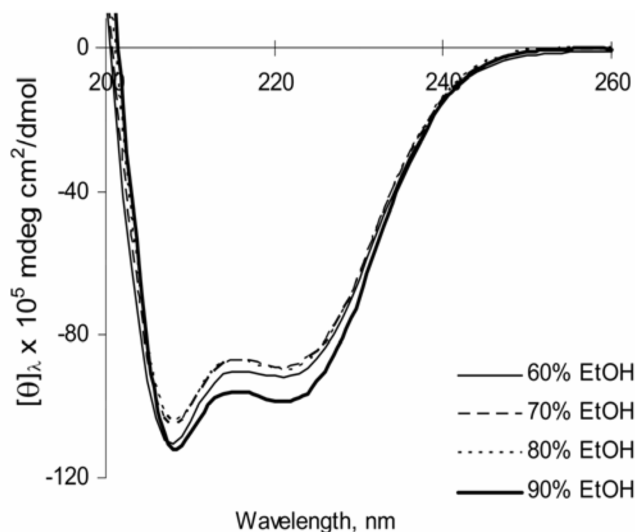

Fig. 5. Near-UV CD spectra of FZ3121 zein at 40 mg/mL in 80% EtOH at 25°C and 70°C.

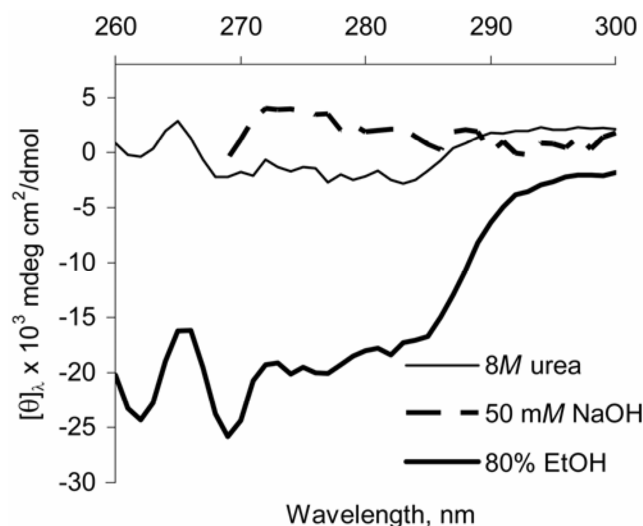

Fig. 7. Near-UV CD spectra of FZ3121 at 40 mg/mL in various solvents at 25°C.

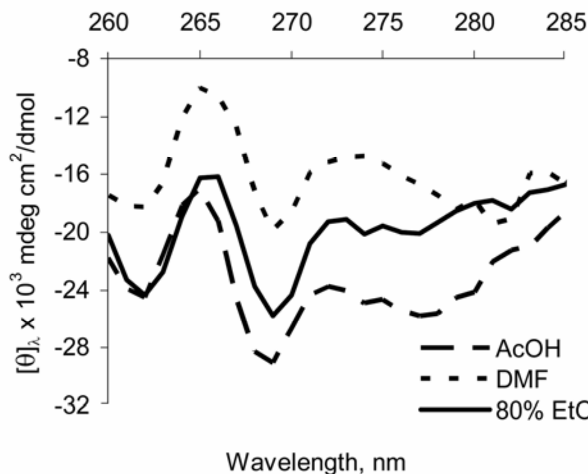

Fig. 8. Near-UV CD spectra of FZ3121 at 40 mg/mL for AcOH and 80% EtOH samples and 34 mg/mL for DMF sample in various solvents at 25°C.

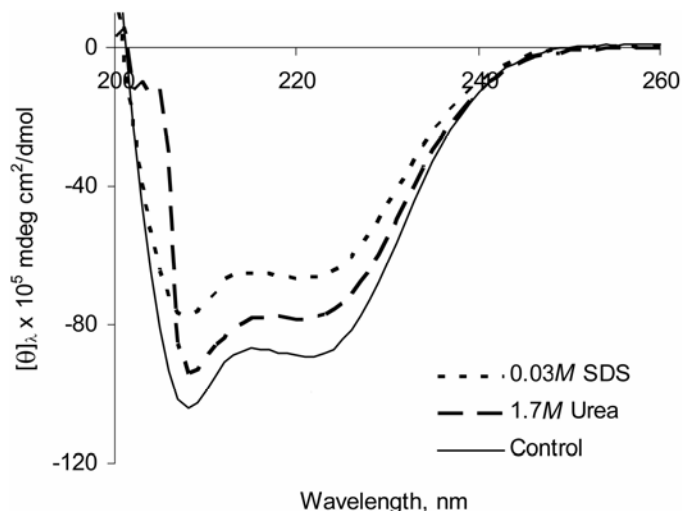

Fig. 9. Near-UV CD spectra of FZ3121 at 40 mg/mL in 80% EtOH with SDS or urea at 25°C.

tion. To determine the extent of degradation, SDS-PAGE was performed on zein that was placed in 50 m*M* NaOH (Fig. 2). While SDS-PAGE is typically not used to determine whether small changes in a protein have occurred, it can be used to detect large amounts of protein degradation. If the very large changes seen in both the near- and far-UV CD spectra were a consequence of protein degradation, then it is expected that some amount of lower molecular weight protein would be visible by SDS-PAGE. The zein base solution was allowed to stand at room temperature for 15, 30, and 60 min. The resulting gel displayed no degradation after these time periods. Therefore, the changes in secondary structure are not due to protein degradation. This result will allow for easier substitution of zein with electrophilic reagents such as acetic anhydride at high pH, with the confidence that the majority of the primary structure will be preserved. The resulting product could be isolated by reducing the pH or by dialysis.

DMF, acetic acid, and 8*M* urea in water cannot be used as solvents for determination of far-UV CD spectra as the solvents interfere with the signal. However, these solvents can be used in near-UV CD experiments. Use of acetic acid or DMF as solvent gave small differences in the near-UV CD spectra of zein (Fig. 8). In AcOH, the absolute magnitude of $[\theta]_\lambda$ at 269 nm increased by 11%. For DMF, the absolute magnitude of $[\theta]_\lambda$ at 269 nm is 23% lower than 80% EtOH. Dissolution of zein in 8*M* urea gave larger changes in tertiary structure, 90% reduction in absolute magnitude of $[\theta]_\lambda$, (Fig. 7) indicative of denaturation.

SDS and urea are two common protein denaturants. To determine the impact of these reagents on zein secondary structure, these reagents were added to 80% EtOH solutions of zein at 0.03*M* and 1.7*M*, respectively (Fig. 9). The addition of 1.7*M* urea to the zein solution does not significantly affect the secondary structure of the protein, giving 10% reductions in the absolute magnitude of $[\theta]_\lambda$. High concentrations of urea in water cannot be used as solvent for far-UV CD due to solvent interference.

Using 0.03*M* SDS in 80% EtOH as solvent shows a reduction in the α-helical character of protein, with a 20% reduction in the absolute magnitude of $[\theta]_\lambda$. When this zein SDS solution is examined in the near-UV, the spectrum is not significantly different than control. The effects of SDS on protein structure are well known, however near-UV CD measures the local environment of the aromatic residues of a protein where the effects of SDS may be different.

Previous studies found that SDS had only a minor effect on intrinsic viscosity, which is related to the mean radius of gyration and is also a function of tertiary structure (Wang et al 2004).

CONCLUSIONS

CD is not sensitive enough to identify differences in zein produced by the same manufacturer, but independently gives differences in another test, so it can be used to help discern differences in zein from different producers. Varying the EtOH fraction in the EtOH and water solutions has little effect on the secondary structure of the protein. Solutions of zein in IPA have more α-helix than the same solutions in either EtOH or MeOH. Use of DMF or AcOH produced small changes in the near-UV CD spectra of zein, suggesting small changes in tertiary structure. Dissolving zein in 8*M* urea produced large changes in the tertiary structure of zein. A zein solution in 50 m*M* NaOH has a predominantly random secondary structure and displays large changes in the near-UV CD spectra. These changes in the CD spectra are not due to significant protein degradation. When an aqueous EtOH zein solution is heated, a reduction in α-helical content and changes in tertiary structure are observed. On cooling this solution, the original structure predominantly returns. Using low levels of either SDS, comparable to that used in an SDS-PAGE test, or 1.7*M* urea in 80% EtOH and water did not produce large changes in the secondary structure of zein. Zein has a very stable structure that requires a high concentration of denaturants, elevated pH, or elevated temperature to produce large structure changes.

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