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Understanding the Dissolution of α-Zein in Aqueous Ethanol and Acetic Acid Solutions

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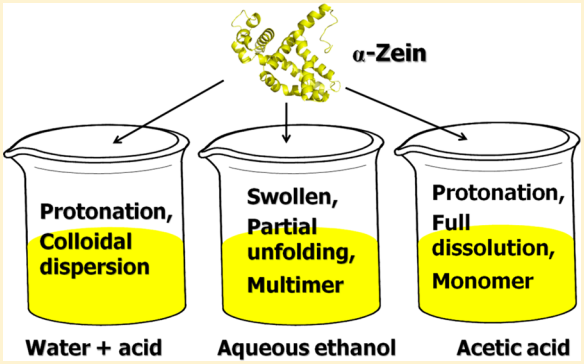
Supporting Information

ABSTRACT: Zein is a corn prolamin that has broad industrial applications because of its unique physical properties. Currently, the high cost of extraction and purification, which is directly related to the dispersion of zein in different solvents, is the major bottleneck of the zein industry. Solution behaviors of zein have been studied for a long time. However, the physical nature of zein in different solvents remains unclear. In this study, small-angle X-ray scattering (SAXS), static light scattering (SLS), and rheology were combined to study the structure and protein−solvent interaction of α-zein in both acetic acid and aqueous ethanol solutions. We found that the like-dissolve-like rule, the partial unfolding, and the protonation of zein are all critical to understanding the solution behaviors. Zein holds an elongated conformation (i.e., prolate ellipsoid) in all solutions, as revealed from SAXS data. There is an "aging effect" for zein in aqueous ethanol solutions, as evidenced by the transition of Newtonian rheological profiles for fresh zein solutions to the non-Newtonian shear thinning behavior for zein solutions after storage at room temperature for 24 h. Such shear thinning behavior becomes more pronounced for zein solutions at higher concentrations. The SLS results clearly show that acetic acid is a better solvent to dissolve zein than aqueous ethanol solution, as supported by a more negative second virial coefficient. This is majorly caused by the protonation of the protein, which was further verified by the dissolution of zein in water (a nonsolvent for zein) with the addition of acids.



INTRODUCTION

Zein is an important natural storage protein from corn kernels that has attracted abundant interests and was once regarded as the most versatile material in the 1950s before the epoch of synthetic polymers.[1,2] To satisfy the increasing demand of environmentally friendly materials in recent years, the industrial value of zein was reevaluated, and numerous scientific studies focusing on zein have been published. The unique features of zein, including the insolubility in water, resistance to grease and microbial attack, glossy appearance, and large availability, which make applications of zein in various fields have countless commercial interests, such as a natural supply of fibers, foods, pharmaceuticals, neutraceuticals, and plastics, etc.,[3,4] and as specific delivery systems for hydrophobic compounds.[5−7] With the help from the advances in experimental methodologies and techniques, a thorough understanding of zein solutions is achievable and also critical not only for the efficient extraction and purification but also for the development of novel zein products.

The selection of the right solvent is important for the extraction and purification of zein as well as controlling the phase behavior and the orientation of zein during its self-assembly. Generally, there are three types of solvents for zein: primary, secondary, and tertiary solvents, which have different impacts on the stability of zein solutions.[1] Typically, primary solvents have a better performance than secondary ones to dissolve zein.[8] Moreover, even in commonly used aqueous ethanol solvents, different ratios of ethanol in water can result in complex phase behaviors,[9−11] different rheological properties,[12,13] different sizes of assembled nanoparticles,[14] and different performances as a biomaterial for cell proliferation.[15] The complexity of zein in aqueous ethanol solutions may be related to the similarity in the polarity of protein and solvent, (e.g., the like-dissolves-like rule). Recent reports have also shown that the zein films prepared from acetic acid and aqueous ethanol solutions have different surface morphologies.[16] Acidic or basic treatments also have a great influence on the properties of zein particles,[17] and zein has a stronger binding affinity to surfaces coated with −COOH groups than with $-CH_3$ groups.[18] All of these observations indicate that the aggregation and conformation of zein are affected not only by the solvent polarity but also by other factors, such as the surface charge of proteins. A clear understanding of the mechanism and

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driving force of the assembly and conformation of zein in different solvents is necessary.

α-Zein is the major component of zein, which has two subtypes, Z19 and Z22, named according to their molecular weights. It is distinct from most globular proteins because of its water insolubility, which is determined by the sequence and structure of zein. According to the native structure model, more than half of the solvent-accessible surface of zein was occupied by hydrophobic residues.[19] The structure and conformation of α-zein in various solutions have been extensively studied.[20−24] Despite the bifurcations, the consensus is that α-zein has an elongated conformation enriched by an α-helix secondary structure. However, the knowledge on how the conformation of zein changes with external factors, such as temperature, concentration, and various solvents, is still limited. Selling et al. found that zein in different fractions of ethanol in aqueous solutions or in acetic acid has a minor impact on the secondary structure based on circular dichroism (CD) measurements.[25] We recently found that zein in acetic acid behaved like polyelectrolytes dissolved in good solvents, according to the concentration-dependent scaling relationships.[19] Whether these behaviors are special for zein in polar protic solvents or widely exist in other systems remains unclear.

In this work, we used a combination of small-angle X-ray scattering (SAXS), rheology, static light scattering (SLS), and UV/vis spectroscopy to study the zein solution properties using aqueous ethanol and acetic acid as solvents. We investigated the conformation of zein as impacted by solvent chemistry and protein concentration and tentatively interpreted the self-assembly of zein in different solutions using the framework of colloidal interaction forces.

EXPERIMENTAL METHODS

Materials. Zein was purchased from Wako Pure Chemical Industries, Ltd. (Lot: TSQ5087; Tokyo, Japan). It has a near equal amount of Z19 and Z22, as characterized in previous work.[26] Acetic acid (ACS reagent grade, >99.7%), sulfuric acid, and hydrochloric acid were purchased from Sigma-Aldrich, Inc. (St. Louis, MO), ethanol (95%) was purchased from Fisher Scientific, Inc. (Pittsburgh, PA), and Milli-Q (18.3Ω) water was used in all experiments.

Small-Angle X-ray Scattering (SAXS) Experiments. The SAXS experiments were conducted at beamline 18-ID of the Advanced Photon Sources at Argonne National Laboratory. The solutions were loaded into the flow cell, which is a cylindrical quartz capillary 1.5 mm in diameter equipped with a brass block, at a constant rate (10 μL/s) during X-ray exposure (1 s) to minimize radiation damage. Each scattering intensity profile was obtained through the average of 15 measurements at room temperature. Solvent and cell background were subtracted using the standard procedure designed for the beamline.

The scattering intensity of SAXS for an isotropic and monodisperse system can be expressed as

$$I(q) = n_p(\delta_p - \delta_s)^2 v^2 P(q) S(q) \quad (1)$$

where n_p is the number of proteins per unit volume, δ_p and δ_s are the electron densities of protein and solvent, respectively, and v is the specific volume of protein, which can be estimated through the relationship proposed by Fischer et al.[27] $P(q)$ is the form factor of the protein, which is related to the protein conformation, and $S(q)$ is the structure factor to reveal the aggregation behavior of the protein.

According to a number of reports, α-zein normally has an elongated conformation.[23,28] Thus, the form factor of zein can be presented using the prolate ellipsoid model, which is written as

$$P(q) = \int_0^1 \left[\frac{3(\sin u - u\cos u)}{u^3}\right]^2 dx \text{ with } u = qb\left[\left(\frac{ax}{b}\right)^2 + (1-x)^2\right] \quad (2)$$

In the above equation, a and b are the parallel and the perpendicular radii of the equivalent ellipsoid of the protein, respectively. Beyond the conformation of proteins, the aggregation/depletion of proteins upon increasing the concentration in solutions can be described using the effective structure factor,[29] $S(q,C)$, which is written as[30]

$$S(q,C) = \frac{C_0 I(q,C)}{C I(q,C_0)} \quad (3)$$

Here, $I(q,C)$ is the scattering intensity profile from a protein solution with concentration C, and C_0 corresponds to a dilute solution in which protein aggregation is negligible.

In addition, the Kratky and Holtzer plot,[31] Guinier plot,[32] and GNOM[33] calculations were also applied to analyze the dimensions of the protein and protein aggregates in solutions.

Rheological Measurements. The apparent viscosities of zein solutions were collected in steady-shear mode using a strain-controlled Advanced Rheometric Expansion system (ARES) (TA Instruments, New Castle, DE). Solutions were loaded into a cone-and-plate with a 50 mm diameter and a 0.04 rad cone angle. A small amount of mineral oil was used to prevent solvent evaporation from the plate edge during measurements, where a shear rate from 1 to 1000 s^{-1} was applied.

Since the apparent viscosity (η_{app}) is proportional to the true viscosity of solutions when the experimental settings (temperature and geometry of sample cell) were fixed according to the Kramer equation,[34] the relative viscosity (η_r) of a solution can be directly obtained through $\eta_{app}/\eta_{s,app}$, with $\eta_{s,app}$ the apparent viscosity of the solvent. On the other hand, it has been reported that zein showed the "aging effect" in various solvents, including aqueous ethanol.[13,35,36] We also observed the syneresis of zein in aqueous ethanol solution, but such a phenomenon did not occur in acetic acid and aqueous acidic solutions during storage. Therefore, the viscosities of zein aqueous ethanol solutions in freshly prepared conditions (within 3 h after sample preparation) and after 24 h of incubation at room temperature were systematically measured.

Static Light Scattering (SLS) Measurements. SLS measurements were conducted on a BIC-200SM Goniometer and Light Scattering System (Holtsville, NY) equipped with a 15 mW He−Ne laser at a wavelength (λ) of 632.8 nm, and a thermostatic device that precisely maintained the temperature at 25 °C. The intensity of the scattered light was collected at various angles with respect to the incident beam. The scattering intensity as a function of the scattering angle (θ) and the concentration of protein can be described by the Zimm equation[37]

$$\frac{KC}{\Delta R(\theta, C)} = \left(1 + \frac{q^2 R_{gz}^2}{3}\right)\left(\frac{1}{M_w} + 2A_2 C\right) \quad (4)$$

Here, $K = [4\pi^2 n_0^2 (dn/dC)^2]/[N_A \lambda^4]$ is a constant for given solutions with a constant refractive index increment (dn/dC), N_A is the Avogadro constant, and n_0 and n are the refractive indexes of pure solvent and solution, respectively. $q = 4\pi n_0 \sin(\theta/2)/\lambda$ is the wave vector, M_w is the molecular weight, R_{gz} is the z-average of the gyration radius of protein, and A_2 is the second virial coefficient. Here, A_2 is an important physical parameter to directly evaluate the solvent quality. A more negative value suggests that the solvent has better quality to dissolve the protein.

Prior to the SLS measurements, all samples were filtered using a 0.2 μm PTFE syringe filter. Protein solutions with concentrations up to 8.0 mg/mL were used to calculate the refractive index increment. A Zimm plot was then generated by plotting $KC/\Delta R(\theta, C)$ against $\sin^2(\theta/2) + \delta C$ with a constant $\delta = 15$ based on the variables used in the measurement. The intensity measurements were performed from 45° to 90°, regarding to an even distribution of q^2. Through the Zimm plot, the protein molecular weight, the z-average gyration radius in dilute solutions, and the second virial coefficient were obtained.

UV Spectroscopy. We prepared three saturated zein solutions for UV−vis adsorption spectrum measurements. These three solutions used 5.0 M sulfuric acid, acetic acid, and hydrochloric acid, respectively, as the solvents. Superfluous zein was added to these acidic solvents and stirred by a Vortex mixer (VWR) for 2 min. After 24 h of magnetic stirring, the solutions were filtered through 0.2 μm PTFE syringe filters. The resulted zein solutions were then diluted three times with the corresponding acids to ensure the UV absorption in the proper range. After subtracting the blank using the corresponding acids, the UV absorption spectra of zein dissolved in different acidic solutions were collected with the wavelength ranging from 200 to 800 nm.

RESULTS

Conformation of Zein in Dilute Solutions. Figure 1 shows the scattering intensity profiles of zein in three different solvents. The fractal dimension in the intermediate q range

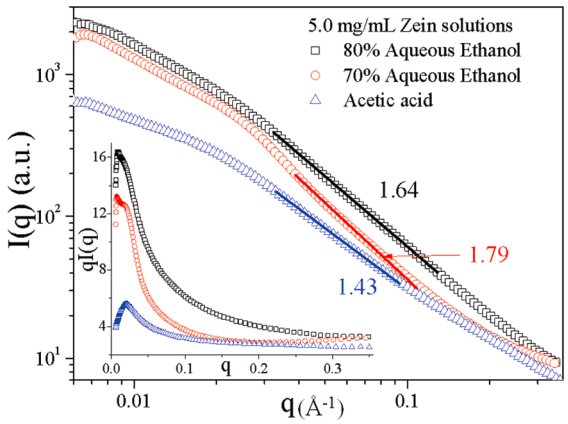

Figure 1. Small-angle X-ray scattering (SAXS) profiles of 5.0 mg/mL α-zein in 80% aqueous ethanol (squares), 70% aqueous ethanol (circles), and acetic acid (triangles). The solid lines show the fractal region in scattering profiles, and the insets are the corresponding Holtzer plots.

provides the contour shapes of proteins in solutions. It is 2 for a random coil with an isotropic Gaussian distribution, and 1 for a rodlike distribution. The fractal dimension varied from 1.43 in acetic acid to 1.79 in 70% aqueous ethanol solutions (v/v), suggesting that α-zein always takes an elongated conformation in these solutions. The scattering profile was then presented using Kratky and Holtzer plots. The Kratky plot (data not shown) did not show any peaks that corresponded to a compact domain for the folded or partially folded structure in proteins. However, the Holtzer plots[38] presented typical profiles, which further suggested that α-zein preferred rodlike conformations in all three dilute solutions.

The isometric prolate ellipsoid model was used to fit the scattering data using eq 2 (shown in Figure 2). The dimensions

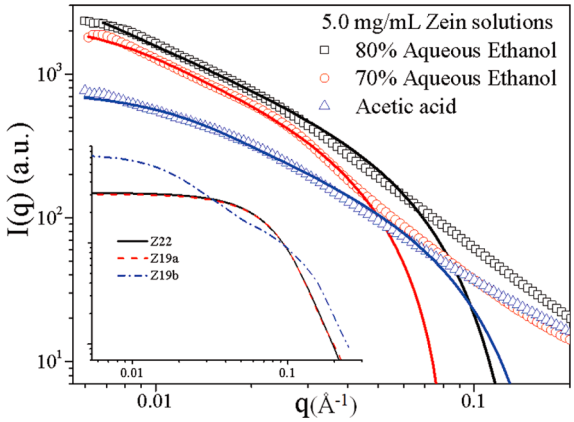

Figure 2. Small-angle X-ray scattering (SAXS) profiles of 5.0 mg/mL α-zein in 80% aqueous ethanol (squares), 70% aqueous ethanol (circles), and acetic acid (triangles) fitted by the ellipsoid model. The solid lines are the best fits of the ellipsoid model, and the inset shows the scattering intensity profile calculated using CRYSOL based on the three structure models (Z22, Z19a, and Z19b) of α-zein.

of α-zein are 21.9 × 3.9 × 3.9 nm^3, 33.8 × 4.4 × 4.4 nm^3, and 22.4 × 2.3 × 2.3 nm^3 in 80% aqueous ethanol, 70% aqueous ethanol, and acetic acid solutions, respectively. The volume of α-zein in 70% aqueous ethanol is almost double of that in the other two solvents, suggesting that α-zein tends to form a dimer in 70% aqueous ethanol solution. It also suggests that a lesser amount of zein was dissolved in 70% than that in 80% aqueous ethanol solutions. This agrees with a recent report that increasing the ethanol content in aqueous ethanol improves the solvent quality for zein.[13] Zein tends to aggregate more in 70% than in 80% aqueous ethanol solution, which is also consistent with the observation by Kim and Xu.[39] The axial ratio from ~5.6:1 to ~9.7:1 of α-zein is within the range of most previous reports.[20,23,24,40]

Furthermore, on the basis of the three atomic structure models of α-zein, Z22, Z19a, and Z19b, in a previous report,[19] the theoretical scattering profile was calculated using CRYSOL,[41] and the results are shown as an inset in Figure 2. Z22 and Z19a are folded, whereas Z19b is in an extended conformation. The overall scattering profiles of α-zein in the three dilute solutions are closer to the extended conformation, suggesting that zein in these dilute solutions is most likely unfolded with less similarity to their native folds.

Concentration Dependence of Zein Aggregate and Conformation. The aggregation of proteins upon increasing the concentration can be obtained from the effective structure

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factor. Complementarily to previous SAXS studies related to zein in 70% aqueous ethanol[20] and acetic acid[19] solutions, we majorly focused on zein in 80% aqueous ethanol solutions. The effective structure factors of zein in 80% aqueous ethanol solution were calculated according to eq 3 and are shown in Figure 3, by referring to the scattering intensity profile at C_0 = 1

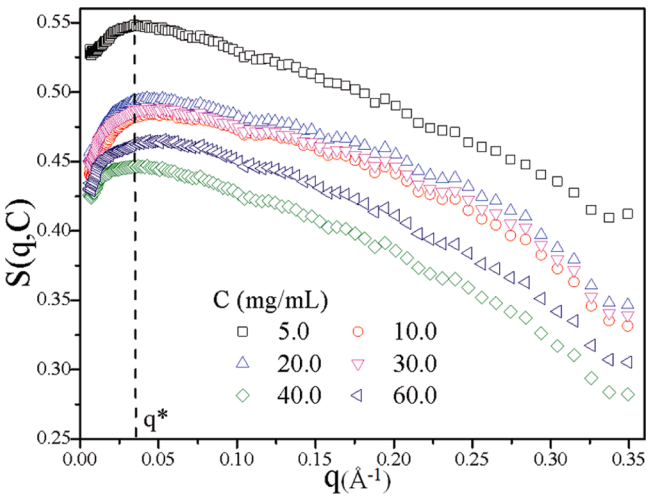

Figure 3. Effective structure factors of α-zein in 80% aqueous ethanol solutions at different concentrations: 5 mg/mL (squares), 10 mg/mL (circles), 20 mg/mL (point-up triangles), 30 mg/mL (point-down triangles), 40 mg/mL (diamonds), and 60 mg/mL (point-left triangles).

mg/mL dilute solution. An upward rising peak at $q^* \sim 0.034$ Å^{-1} always shows up, and the peak position is independent of concentration. This peak indicates the attractive interaction among proteins, and the increase of concentration eventually causes aggregation of proteins. It appears at finite q, which corresponds to the correlations between proteins,[42] and the correlation length is 18.5 nm by converting the peak position using $2\pi/q^*$. By comparing the upward peak in aqueous ethanol solutions and the concave peak corresponding to 38.1−42.0 nm in acetic acid,[19] one finds that proteins have long-range repulsion in acetic acid solution, but attraction and aggregation in aqueous ethanol solution. Furthermore, by assuming that proteins are in a monomer state and evenly dispersed in solution, we evaluated the average correlation length among proteins (the average separation of proteins) through $2(3M_w/4\pi N_A C)^{1/3}$ by taking 20.8 kDa as the molecular weight of α-zein (M_w) based on our previous study.[19] The correlation length in 80% aqueous ethanol solutions corresponds to a homogeneous solution with a concentration of 53 mg/mL and is independent of the concentration of zein. It suggested that proteins were heterogeneously dispersed in the 80% aqueous ethanol solution and partially present as dimers or multimers.

On the basis of Guinier plots for globular and rodlike particles, the gyration radius (R_g) and the cross-sectional radius (R_c) of α-zein in 80% and 70% aqueous ethanol solutions were obtained and are plotted against zein concentration in Figure 4. It can be seen that the size and the axial ratio of zein have no obvious dependence on concentration in the 80% aqueous ethanol solutions, and the R_g and the axial ratio slightly decrease with the increase of concentration in the 70% aqueous ethanol solutions. The axial ratio calculated through $4/3(R_g/R_c)^3$ is around 8.6, which is close to that obtained from dilute solutions. Compared with the concentration dependence of

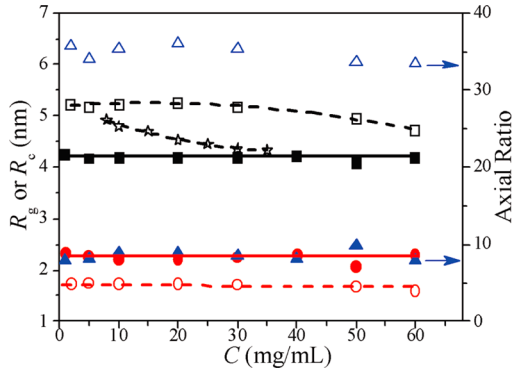

Figure 4. Sizes and the axial ratios of α-zein in 80% (solid symbols) and 70% (empty symbols) aqueous ethanol solutions plotted against α-zein concentration. Squares, circles, and triangles represent the gyration radius (R_g), the cross-sectional radius (R_c), and the axial ratio, respectively. The star symbol is the R_g of α-zein in 70% aqueous ethanol solutions reported by Matsushima et al.[20] Lines are used to guide the eyes.

protein size in acetic acid solutions, as reported in previous work,[19] where the R_g decreased as the concentration increased and obeyed the similar scaling law as polyelectrolytes dissolved in good solvents, one may conclude that aqueous ethanol is a θ solvent for zein, because polymer sizes are not obviously dependent on the polymer concentration in θ solvents.[43] This is reasonable because it has been reported that acidic acid as a primary solvent has a much better performance than aqueous ethanol as a secondary solvent to dissolve zein.[8] By comparing the size of zein in 70% with that in 80% aqueous ethanol solutions, the R_g of zein was found to be 1.2−1.3 times larger than that in 80% aqueous ethanol solution, suggesting that zein tended to form dimers in the 70% aqueous ethanol solutions. The axial ratio in 70% is around 4 times larger than that in 80% solution, suggesting that the zein dimer formed through packing on the principal axis with a more elongated conformation of the zein monomer. The size of zein in 70% aqueous ethanol solutions is close to that in the report using SAXS by Matsushima et al.,[20] though the size shows less decrease with concentration than their measurement. The disagreement in the size and concentration dependence may stem from the difference in zein samples used.

Viscosity of Zein Solutions. The rheological measurements of zein in 80% and 70% aqueous ethanol solutions (fresh and incubated) are shown in Figure 5. All freshly prepared solutions show Newtonian behavior with plateau viscosities, which were taken as the average apparent viscosities of the corresponding solutions. For incubated solutions (after 24 h storage at room temperature), shear thinning could be observed from concentrated solutions, in agreement with numerous reports.[12,13,17,35] The shear thinning phenomenon in 80% aqueous ethanol is less prominent than that in 70% aqueous ethanol solution, suggesting less aggregation of zein in the former solvent.[12] When shear thinning is prominent, the zero shear rate viscosity (η_0) is derived through the approximation based on the Cross model,[44−46] which is written as

$$\eta = \eta_\infty + \frac{\eta_0 - \eta_\infty}{1 + bPe} \quad (5)$$

Here, η and η_∞ are the apparent viscosity and viscosity of solution at infinite shear rate, respectively. b is a constant, and the Péclet number, Pe, equals $a^3\eta_s\gamma/k_BT$,[47] with a the

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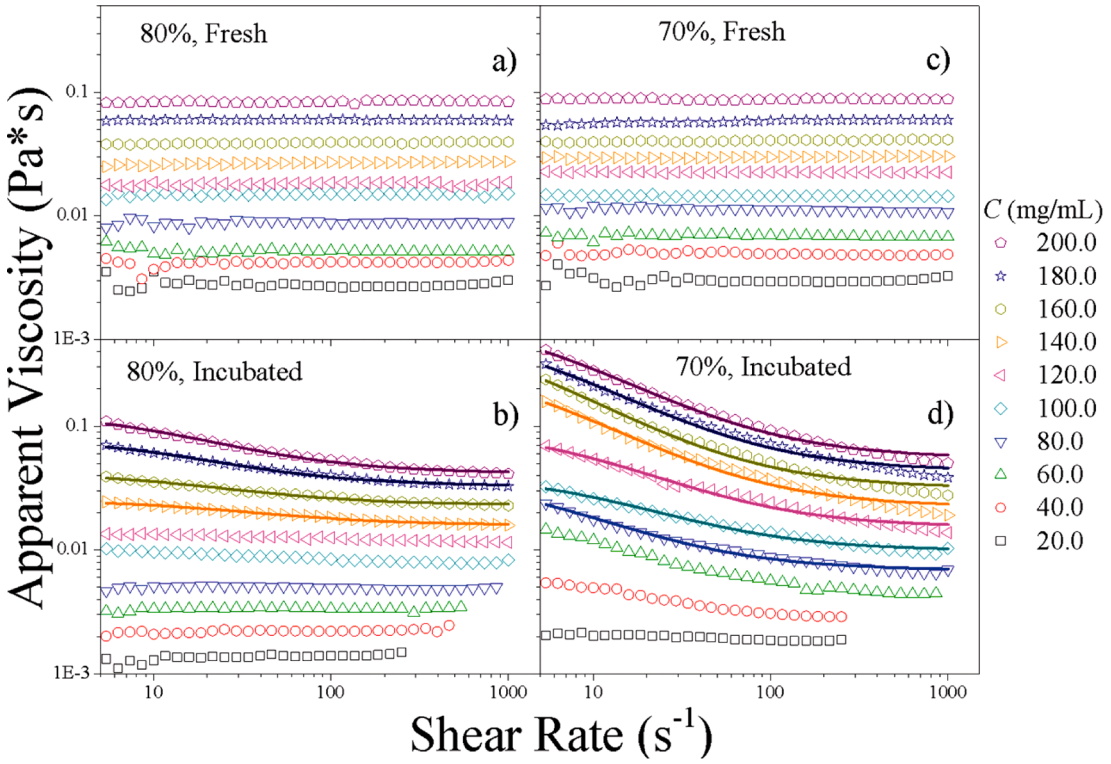

Figure 5. Viscosities of α-zein versus shear rate in 80% (a, b) and 70% (c, d) aqueous ethanol in freshly prepared and incubated (storage for 24 h) solutions. The solid lines in (b) and (d) are the best fits with R^2 larger than 0.99 using eq 5.

hydrodynamic radius of the protein, η_s the viscosity of the solvent, γ the shear rate (s^{-1}), k_B the Boltzmann constant, and T the absolute temperature. For zein solutions with plateau viscosities (e.g., in freshly prepared aqueous ethanol solutions, acetic acid solutions, and dilute incubated aqueous ethanol solutions), we hypothesize that the zero shear rate viscosities do not significantly deviate from the plateau viscosities. Therefore, solution viscosity was used to represent both zero shear rate viscosity and the average plateau viscosity without further distinguishing them in the following sections.

The concentration dependence of the relative viscosity (η_r), which was normalized by the apparent viscosity of the corresponding solvent, is shown in Figure 6. It can be fitted using the relationship proposed by Fu et al.,[12] which is written as $\ln \eta_r = A^*C$, with A the slope to present the protein concentration dependence. The viscosity from freshly prepared solutions has minor differences in either 70% or 80% aqueous ethanol solutions, and they can be well fitted by the half-logarithm linear relationship with a slope close to 0.019 mL/mg. The upper limit of the slope can trace on the viscosity from incubated (stored for 24 h) aqueous ethanol solutions and acetic acid solutions, with a slope of 0.028 mL/mg. However, the relative viscosity from concentrated zein in 70% aqueous ethanol solutions failed to fall in this region, partially due to strong shear thinning. The slope range from 0.019 to 0.028 mL/mg is larger than that reported by Fu et al.[12] The source and purity of zein could be the major cause for the discrepancy, as pointed out by Selling et al.[35] Alternatively, we also plotted the specific viscosity of zein solutions ($\eta_r - 1$) against zein concentration, as shown in Figure S1 in the Supporting Information. The viscosity−concentration scaling was converged at 2.85 for all fresh solutions and agreed with the one (2.70) reported recently.[19]

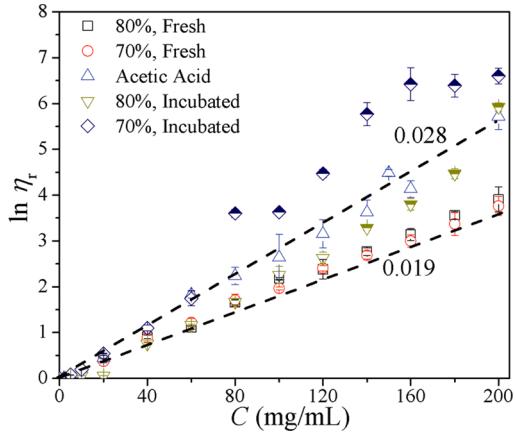

Figure 6. Relative viscosities of α-zein in different solvents plotted against α-zein concentration: freshly prepared 80% aqueous ethanol (squares), freshly prepared 70% aqueous ethanol (circles), acetic acid (point-up triangles), incubated 80% aqueous ethanol (point-down triangles), and incubated 70% aqueous ethanol (diamonds). The half-filled symbols represent the relative viscosity derived from the zero shear rate viscosity divided by the viscosity of solvent, while the dashed lines show the slopes in which most of the solution viscosities are located.

Static Light Scattering (SLS) Measurements of Zein Solutions. The SLS measurements of zein in 70% aqueous ethanol and acetic acid solutions are presented in Figure 7, and the fitting results from Zimm plots are summarized in Table 1. The refractive index of the solutions was found to linearly increase with the protein concentration, which ensures that the Zimm plot is reliable. With the best fit refractive index increment constant, extrapolation of the scattering intensities from finite dilute solutions are well convergent at the limits of C

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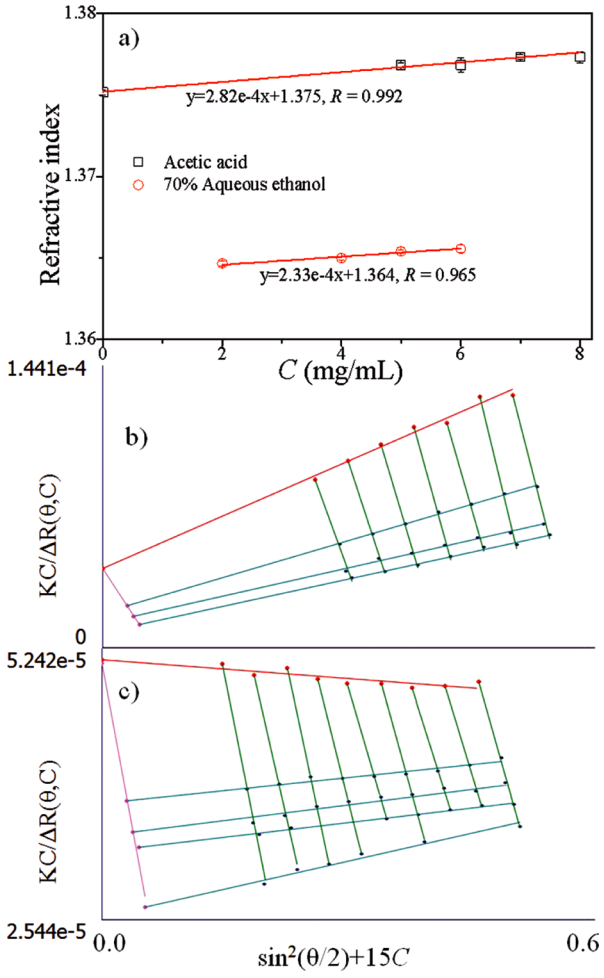

Figure 7. Static light scattering (SLS) measurements of α-zein solution properties: (a) the plots of refractive indexes versus α-zein concentration in acetic acid (squares) and 70% aqueous ethanol (circles); (b) Zimm plot for α-zein in 70% aqueous ethanol; (c) Zimm plot of α-zein in acetic acid solution.

$\rightarrow 0$ and $\theta \rightarrow 0$. The molecular weights upon extrapolation were 20–22 kDa, which is consistent with the average molecular weight, 20.8 kDa, of α-zein with almost equal amounts of Z19 and Z22.[19] The z-average radius of gyration (R_{gz}) is much larger than the radius of gyration from SAXS analysis, which may be due to the rodlike nature of zein conformation or the formation of large protein aggregates (e.g., dimers, tetramers, etc.).[28,48] It is reasonable because, on one hand, R_{gz} is emphasized more on large particles than R_g from SAXS, according to its definition, and, on the other hand, the nonuniform distribution of zein in aqueous ethanol solutions has been reported from a rheological study.[12] Most importantly, we found that the second virial coefficient in acetic acid solution is more negative than that in 70% aqueous ethanol solution, which is a direct proof for the conclusion derived from solution stability made by Evans, that the single solvent is much better than aqueous ethanol systems.[8]

UV Spectroscopy of Zein in Acidic Solutions. We tentatively speculated that the better quality for acetic acid than aqueous ethanol to dissolve zein is from additional protonation of the protein in the former solvent. To address this point, the UV absorption spectroscopic measurements were carried out to determine whether zein could be dissolved in its nonsolvent, water with the presence of acid. The UV absorption spectra in the wavelength range of 250–440 nm are shown in Figure 8.

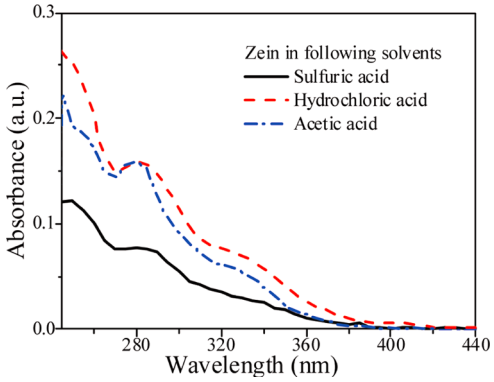

Figure 8. UV absorption spectra of 5.0 mol/L α-zein in different acid solutions: sulfuric acid (solid line), hydrochloric acid (dashed line), and acetic acid (dashed-dotted line).

The peak at around 280 nm corresponding to the aromatic rings in the protein, which is normally used to determine the concentration of the protein, can be clearly observed. It suggests that zein can be dissolved in water with the addition of acids, further proving that protonation of zein could help zein dissolve in its nonsolvent, water.

DISCUSSION

First, it is of interest to know how the conformation of α-zein changes with different solvents and concentrations. In dilute solutions, compared to previous reports of zein in acetic acid and 70% aqueous ethanol solutions,[19,20] the size of zein decreased in the order of acetic acid, 70% aqueous ethanol, and 80% aqueous ethanol. The sizes of α-zein in all dilute solutions are larger than that of the folded structure models of α-zein, which is around 2.3 nm.[19] It suggests that the protein was at least partially unfolded in these solutions. According to the CD measurements of zein in these solutions,[25] the secondary structure of zein in aqueous ethanol and acetic acid has no significant difference. The unfolding most likely occurs in the coil region. With the increase of zein concentration, α-zein in acetic acid behaves like polyelectrolytes dissolved in a good solvent and the size slightly decreases, whereas in 80% and 70% aqueous ethanol solutions, the size is almost independent of concentration. The size of zein in the former solvent is larger than that in aqueous ethanol, suggesting that α-zein is more unfolded or swollen in the presence of acetic acid.

Table 1. Summary of Molecular Weights, z-Average Gyration Radii, and the Secondary Virial Coefficients of α-Zein in 70% Aqueous Ethanol and Acetic Acid Obtained from Zimm Plots by Static Light Scattering Measurements

solvents	$M_{w,\,C\rightarrow 0}$ (kDa)	$M_{w,\,\theta\rightarrow 0}$ (kDa)	A_2 (mL*mol/g^2)	R_{gz} (nm)
70% aqueous ethanol	22.2 ± 3.5	22.2 ± 3.1	$-5.5 \pm 3.5 \times 10^{-3}$	108 ± 14
acetic acid	20.1 ± 3.0	20.5 ± 2.1	$-12.2 \pm 2.3 \times 10^{-3}$	126 ± 16

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Second, there are three different interpretations for the effects of zein concentration on the solution viscosity. Fu et al.[12] found an exponential increase of viscosity against zein concentration in aqueous ethanol, Zhang et al.[17] observed shear thinning behavior of concentrated zein in 70% aqueous ethanol solutions, and Selling et al.[35] also observed both the exponential increase against concentration and the shear thinning behavior for zein dissolved in *N*,*N*-dimethylformamide (DMF). Our previous finding[19] indicated that the viscosity had two typical scaling regions analogous to dilute and semidilute polyelectrolyte solutions. These different dissolution behaviors of zein in different solvents may be directly related to the different morphologies of zein films prepared from either acetic acid or aqueous ethanol solutions.[16] However, we did not find a significant difference in the structure according to SAXS results. The aging effect in rheological measurements suggested that the different phase of zein particles obtained from aqueous ethanol solutions as reported by Wang and Padua[9] was not due to the difference in the structure and the association of protein.

Interestingly, from the concentration-dependent protein conformation and solution viscosity, we concluded that zein behaved like polyelectrolytes in good solvent (i.e., acetic acid) and like a polymer dissolved in a θ solvent (i.e., aqueous ethanol). It may result from various interactions at the molecule level. For example, the hydrophobic/hydrophilic nature of the protein surface plays a role under the "like-dissolve-like" rule, partial unfolding of zein may change the solvent-accessible area and hydration of the zein surface, and protein protonation by association with a protic solvent may also prohibit protein aggregation to allow better dissolution. Overall, acetic acid has better solvent quality than aqueous ethanol systems to dissolve zein, as viewed from the second virial coefficient values obtained from the SLS study. Regarding the hydrophilicity of the solvent, the polarity index of water is 9.0, acetic acid is 6.2, and ethanol is 5.2, according to the solvent miscibility table. The hydrophilicity of acetic acid and 70% and 80% aqueous ethanol are all very close. The hydropathy index of zein is 4.70 and 4.86 for Z19 and Z22, respectively, based on the calculated parameters reported by Kyte.[49] Therefore, by taking into account the polarity similarity to zein (i.e., assume that hydrophobic interaction is the major hindrance to prevent zein from being solvated), acetic acid and aqueous ethanol have indistinguishable differences. Therefore, the like-dissolve-like rule should not be the major contributor to the difference in solvent quality. In the second aspect, the partial unfolding can be viewed from the comparison of R_g to that of native structure models. In dilute solutions, R_g in acetic acid is larger than those in aqueous ethanol, suggesting that zein is more unfolded in acetic acid, which provides a larger solvent-accessible area and eventually helps the dissolution of zein. Finally, on the protonation of the protein surface, since it is easier for the −COOH group in acetic acid to release free hydrogen ions than the −OH group in ethanol, protonation of zein in acetic acid (i.e., positive charges in the side chain of arginine, lysine, and histidine residues in zein can be stabilized through salt bridges with acetate) is more common than in aqueous ethanol solutions. The protonation of zein enhanced the solution stability through long-range electrostatic repulsion induced by positive charges on the zein surface and reduction of hydrophobic interaction with the presence of the acetate solvation layer. This speculation can also be used to understand the concentration dependence of the solution viscosity. Solution viscosity has a steeper increase upon increasing the concentration in acetic acid than that in freshly prepared aqueous ethanol solutions. It is reasonable because protonated proteins with long-range electrostatic repulsions are easier to get dynamic arrest, which is the major contributor to the steep increase of solution viscosity against concentration.[50] The importance of protonation in zein dissolution can be clearly understood from the UV absorption spectroscopy, where the addition of acids can dissolve zein into its nonsolvent, water. This also revealed that, in real applications, the addition of acids normally enhanced the water disparity of a prolamin−polysaccharide complex[51] because of the protonation of protein.

The comparison of zein in three kinds of solvents is illustrated in Figure 9. Zein is fully dissolved in acetic acid and behaves like polyelectrolytes. It associated with acetate through salt bridges. It is partially swollen in aqueous ethanol solutions, and the shear thinning in high concentration can be understood as the loss of entropy arising from the rearrangement of swollen particles.[44,52] Zein in 80% aqueous ethanol solution is more dissolved and less polymerized than those in 70% aqueous solutions. The protonation of zein can help stabilize zein in its nonsolvent, water, with long-range electrostatic repulsion, the identical mechanism for colloid dispersion and stabilization.[53]

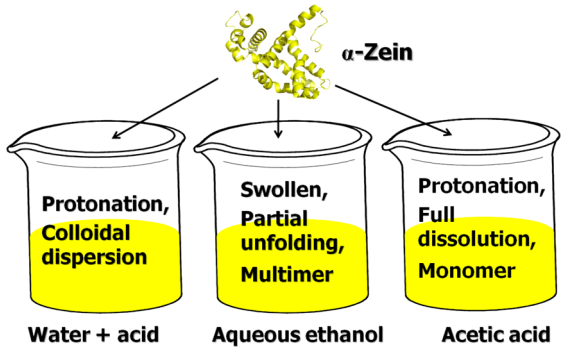

Figure 9. Schematic diagram of α-zein in three different solvents: (left) water + acid; (middle) aqueous ethanol; (right) acetic acid.

CONCLUSION

In summary, we performed SAXS, rheology, and SLS to study solution behaviors of α-zein in acetic acid and aqueous ethanol solutions at different protein concentrations. It was found that, different from α-zein in acetic acid solution, which behaved like polyelectrolytes with typical scaling relationships on the size and viscosity against protein concentration, α-zein behaved like swollen colloids in aqueous ethanol solutions where the size changed negligibly with protein concentration. An elongated conformation of α-zein was consistently observed in all three solvents studied. The aging effect of zein in aqueous ethanol solution has a significant influence on its rheological profiles, especially at higher protein solutions. We also directly verified that acetic acid had a better solvent quality than aqueous ethanol to dissolve zein, according to the values of the second virial coefficient, which further indicated that the higher solubility of zein in acetic acid than in aqueous ethanol was not due to the difference in hydrophobicity of these three solvents. Instead, it was caused by the protonation of acetic acid imposed on zein.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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