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Effect of γ-Zein on the Rheological Behavior of Concentrated Zein Solutions

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ABSTRACT: Zein, the prolamin of corn, is attractive to the food and pharmaceutical industries because of its ability to form edible films. It has also been investigated for its application in encapsulation, as a drug delivery base, and in tissue scaffolding. Zein is actually a mixture of proteins, which can be separated by SDS-PAGE into α-, β-, γ-, and δ-zein. The two major fractions are α-zein, which accounts for 70−85% of the total zein, and γ-zein (10−20%). γ-Zein has a high cysteine content relative to α-zein and is believed to affect zein rheological properties. The aim of this study was to investigate the effect of γ-zein on the often observed phenomena of zein gelation. Gelation affects the structural stability of zein solutions, which affects process design for zein extraction operations and development of applications. The rheological parameters, storage modulus (G') and loss modulus (G''), were measured for zein solutions (27% w/w solids in 70% ethanol). β-Mercaptoethanol (BME) was added to the solvent to investigate the effect of sulfhydryl groups on zein rheology. Modulus data showed that zein samples containing γ-zein had measurable gelation times under experimental conditions, contrary to samples with no γ-zein, where gelation was not detected. Addition of BME decreased the gelation time of samples containing γ-zein. This was attributed to protein unfolding. SEM images of zein microstructure revealed the formation of microspheres for samples with relatively high content of α-zein, whereas γ-zein promoted the formation of networks. Results of this work may be useful to improve understanding of the rheological behavior of zein.

KEYWORDS: *zein, rheology, zein fractions, α-zein, γ-zein*

INTRODUCTION

Zein, the prolamin of corn, is attractive to the food and pharmaceutical industries because of its ability to form edible films.[1,2] It has been investigated for its application in encapsulation,[3] as drug delivery base,[4] and in tissue scaffolding.[5] Zein is actually a mixture of proteins, which can be separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) into α-, β-, γ-, and δ-zein. According to Esen's classification,[6] α-zein has an apparent molecular weight of 21−25 kDa and a smaller fraction at 10 kDa; β-zein, 17−18 kDa; and γ-zein, 27 kDa. Thompson and Larkins's classification[7] has α-zein at 19 and 22 kDa, β-zein at 14 kDa, γ-zein at 16 and 27 kDa, and δ-zein at 10 kDa. Later, Esen[8] recategorized the 10 kDa fraction as δ-zein. α-Zein is the most abundant fraction, accounting for 70−85% of the total zein, followed by γ-zein (10−20%), β-zein (1−5%), and δ-zein (1−5%).[9] All zein fractions are considered to be amphiphilic because they contain both hydrophobic and hydrophilic amino acid residues. However, zein fractions differ in their content of cysteine, which is believed to affect their rheological behavior.

The 19 kDa α-zein contains 240 amino acids including 1−3 cysteine residues.[10,11] The 22 kDa α-zein has 265 amino acids with 1 cysteine.[10,12] By contrast, γ-zein is rich in cysteine. The 16 kDa γ-zein contains 183 amino acids with 12 cysteine residues.[11] The 27 kDa γ-zein has 223 amino acids with 14 cysteine residues.[13] β- and δ-zein also contain higher amounts of cysteine compared to α-zein. β-Zein contains 180 amino acids with 8 cysteine residues.[10,14] δ-Zein has 150 amino acids with 6 cysteine residues.[15] This information is summarized in Table 1. Zein can be dissolved in primary solvents such as pyridine and formic acid[16] and secondary solvents including mixtures of alcohols and water and acetone and water.[17] Among them, aqueous alcohols are the most often used because they are relatively easy to recover.[18]

Table 1. Amino Acid Content of Zein

	α-zein		γ-zein		β-zein	δ-zein
amino acid	19 kDa[10,11]	22 kDa[10,12]	16 kDa[11]	27 kDa[13]	15 kDa[10,14]	10 kDa[15]
total no.	240	265	183	223	180	150
polar (%)	42.65	42.26	51.91	47.53	50.00	33.37
nonpolar (%)	57.35	57.74	48.09	52.47	50.00	66.67
cysteine (%)	0.84	0.38	6.56	6.28	4.44	4.00

Zein is commercially extracted from corn gluten meal (CGM), a byproduct of wet milling. In the process, SO_2 is used to soften the kernels and facilitate the separation of starch.[19] It also breaks disulfide linkages found in β-, γ-, and δ-zein. γ-Zein becomes water-soluble after the bonds are cleaved[20] and is possibly discarded with the steep water. Commercial zein is mostly α-zein.[21] According to Anderson and Lamsal,[22] zein extracted from dry-grind ethanol processes is becoming increasingly available. This type of zein is composed of α-, β-, and γ-zein, possibly because the steep process is not applied. The presence of β- and γ-zein is

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presumed to affect zein rheological properties and lead to gelling.[23,24]

Rheology is important in process design including pumps and other fluid transport devices. It is also critical in the development of applications. Several researchers have studied the rheological behavior of α-zein solutions. Fu and Weller[25] studied the rheology of zein solutions using a Brookfield viscometer. They reported that zein solutions (2−14% w/w in ethanol) showed Newtonian behavior. However, zein solutions were unstable and tended to form gels. Selling and co-workers[24] studied the rheology of zein in *N*,*N*-dimethylformamide (DMF). They suggested that zein viscosity depended on protein content. Fat content was not a significant factor. They also reported that highly concentrated solutions (>15% protein) tended to form gels. Zhang and Wang[26] used an AR2000ex system to measure the storage modulus (G') and loss modulus (G'') of zein in aqueous ethanol at various pH levels. They observed that zein had lower G' and G'' in solutions of higher or lower pH than at a nearly neutral pH of 6.5. Results were attributed to deamidation of glutamine at extreme pH values, which possibly led to low zein aggregation. However, only a limited number of studies on the effect of other fractions on rheology of zein solutions are available.

The potential increase in zein production, related to fuel ethanol manufacture and the development of novel applications, highlights the need for a better understanding of the contribution of the different protein fractions to zein rheology. γ-Zein is the second most abundant fraction. However, its impact on zein properties is still unclear. The aim of this study was to investigate the effect of γ-zein content of zein samples on gelation phenomena. Gelation affects the structure stability of zein solutions, which affects process design in zein extraction operations and in the development of applications.

MATERIALS AND METHODS

Materials. Zein, purchased from Showa Sanyo Co. Ltd. (Tokyo, Japan), was designated S zein. Zein prepared in a pilot plant run by extraction of ground whole corn with 70% ethanol, followed by membrane separation,[27] was obtained from Prairie Gold, Inc. (Bloomington, IL). It was designated P zein. Absolute ethanol was from Decon Laboratories, Inc. (King of Prussia, PA). β-Mercaptoethanol (BME), methanol, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). The SDS-PAGE system including 8−16% Tris-HCl ready gel, Laemmli sample buffer, running buffer, Precision Plus Protein Dual Xtra standard, and Coomassie Blue G-250 was from Bio-Rad Laboratories (Hercules, CA).

Separation of Zein Fractions. Zein (P) was extracted with 90% ethanol to separate α-zein, according to the method of Paraman and Lamsal.[28] The extract was centrifuged for 30 min at 20 °C at 10000*g* (RC5C Sorvall Instruments, Newton, CT). Both pellet and supernatant were dried under a hood at room temperature. Dried samples were kept in a sealed container. The supernatant, rich in α-zein,[28] was labeled α-rich extract. The pellet, rich in β- and γ-zein,[28] was referred to as γ-rich pellet.

SDS-PAGE. SDS-PAGE was conducted to separate and characterize zein fractions of different molecular weights. Zein (1% w/v) was dissolved in 70% ethanol and then diluted 1:2 with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, and 0.01% bromophenol blue). Protein solutions were divided in two groups. For one group 5% BME was added to the sample buffer, whereas the other had no BME added. Solutions were then boiled for 5 min. A 8−16% Tris-HCl ready gel was loaded with 10 μg of each protein preparation. A Precision Plus Protein Dual Xtra standard was used as a molecular weight marker. The running buffer was Tris/glycine/SDS containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3. The gel was run at 200 V for 30 min, fixed for 30 min in fixing solution (40% methanol, 10% acetic acid, and 50% water), and stained overnight with Coomassie Blue G-250. The stained gel was destained with 10% acetic acid and imaged with a Kodak Image Station 440 CF (Eastman Kodak Co., New Haven, CT).

Rheological Measurements. The viscoelastic properties of the various zein samples were analyzed by oscillatory time sweep tests. Oscillatory tests are useful for tracking the setting of gelling systems. This is possible because in the linear G' and G'' regions, the amplitude is small enough so as not to interfere with the microstructure or the setting mechanism.[29] The rheological parameters, storage modulus (G') and loss modulus (G''), of zein dispersions were measured over time with an ARES-G2 rheometer (TA Instruments, New Castle, DE) using a DIN concentric cylinder (bob diameter of 27.7 mm and cup diameter of 30 mm). Zein samples, 27% (w/w), were dispersed with magnetic stirring in 70% ethanol containing three levels of BME (0, 1, and 5%). Samples of 22 mL were placed in the test cup. Prior to running the test, the top surface of the sample was covered by 1 mL of 100% mineral oil (Cannon Instrument Co., State College, PA) to minimize solvent evaporation during the experiment.[30] To determine the appropriate experimental conditions for the rheological test, linear viscoelastic ranges were determined by oscillation amplitude tests carried out at a frequency of 1 Hz with a strain variation from 0.01 to 100%. Results showed that 0.3% strain at 1 Hz could be applied to all samples. Therefore, oscillatory time sweep tests were performed at 1 Hz and 0.3% strain. Each experiment was conducted at 20 °C for a period of 4 h. Data were collected at 30 s intervals.

Scanning Electron Microscopy (SEM). SEM was used to characterize sample microstructure. Zein (1 mg/mL) samples were dissolved in 70% ethanol containing three levels of BME (0, 1, and 5%). Sample solutions were placed under the hood at room temperature to allow for evaporation-induced self-assembly. Dry samples were gold coated (300 Å) before imaging, with an Emitech K575 sputter coater (Ashford, U.K.) to enhance the electrical conductivity of sample surfaces. SEM images were collected with a JEOL6060LV general purpose SEM (Peabody, MA).

RESULTS AND DISCUSSION

SDS-PAGE Analysis. An SDS-PAGE electrogram of S zein, P zein, α-rich extract, and γ-rich pellet is shown in Figure 1. S

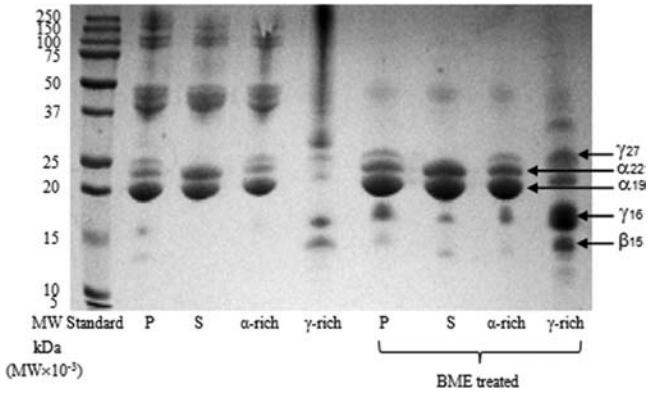

Figure 1. SDS-PAGE of zein samples: P, P zein; S, S zein; α-rich, α-rich extract; γ-rich, γ-rich pellet, with and without BME treatment.

zein shows two major broad bands at 19−22 and 23−24 kDa, which indicate the presence of α-zein.[7,9] Another band appeared at 38−48 kDa, suggesting dimerization. Lighter bands were observed at 88 kDa and higher molecular weight, indicating further oligomerization. Similarly to S zein, P zein showed the dark, broad bands of α-zein at 19−22 and 23−24 kDa. In contrast, light bands at 16 and 27 kDa indicated the presence of γ-zein.[7] Oligomerization bands at 38−48 kDa and above 88 kDa were broad and darker, suggesting a stronger

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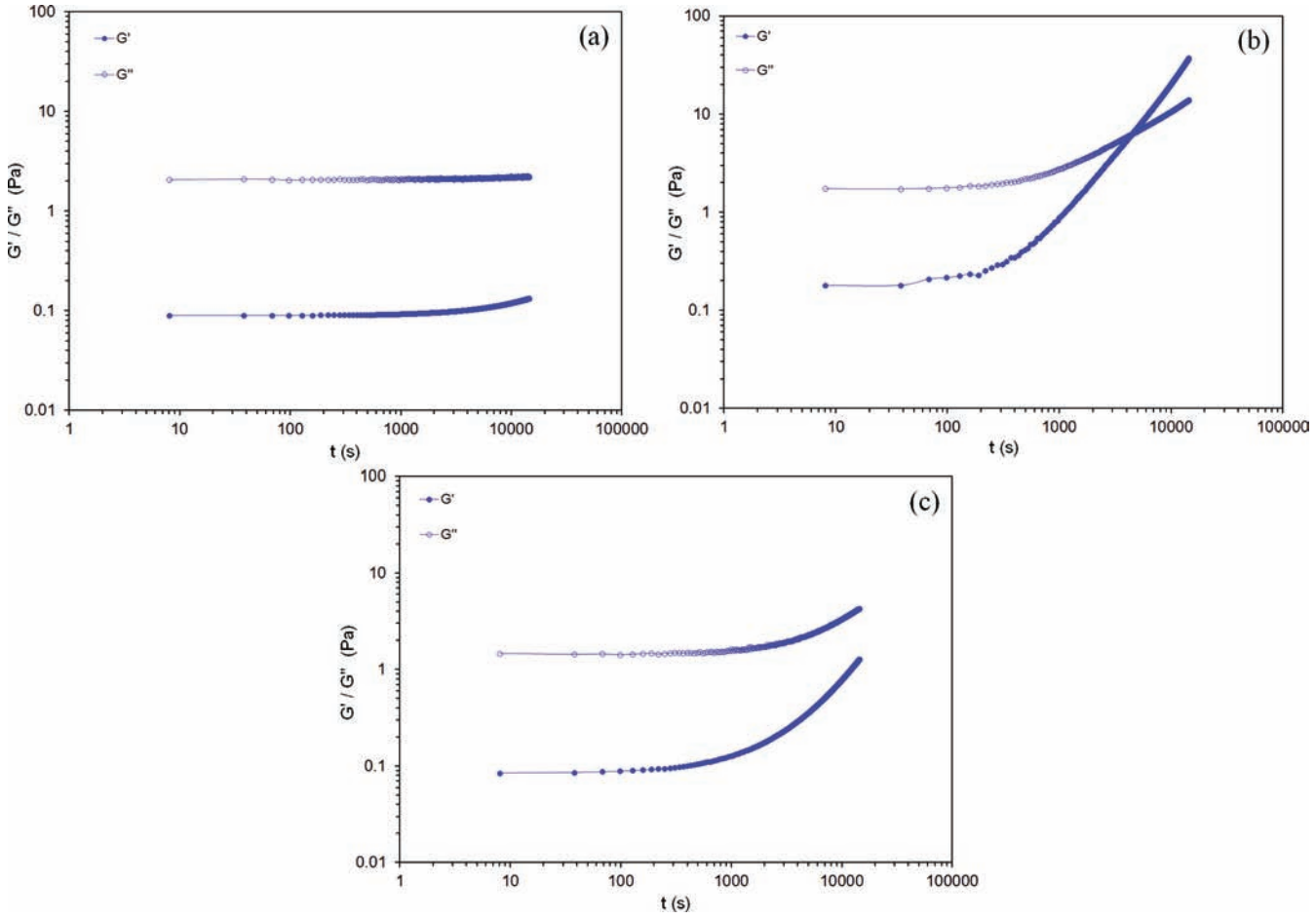

Figure 2. Oscillatory time sweep results of zein solutions, 27% (w/w) in 70% ethanol, run at 1 Hz and 0.3% strain: (a) S zein; (b) P zein; (c) α-rich extract.

tendency for oligomerization than shown by S zein. To investigate the differences between those two samples, P zein was extracted with 90% ethanol to separate out α-zein[28] from the rest of fractions. SDS-PAGE of the α-rich extract showed a pattern similar to that of P zein, but without the 16 kDa γ-zein band. The oligomer bands, 38−48 kDa and above 88 kDa, were somewhat less intense. The residual fraction after extraction, labeled γ-rich pellet, showed bands at 16 and 27 kDa, which were considered to be γ-zein.[7] A band also appeared at 15 kDa, which represented β-zein. A smaller light band appeared at 19 kDa, representing α-zein.[7] Another band was observed at 31 kDa, possibly a dimer. Polymerization bands at very high molecular weight were also present.

BME was added to investigate whether the high molecular weight fractions represented new fractions or aggregates of α-, β-, γ-, and δ-zein. After the addition of BME, bands at 88 kDa and higher molecular weight disappeared for all zein samples. Also, the fraction at 38−48 kDa faded. α-Zein bands were stronger for all samples compared to the patterns before BME treatment. The 16 kDa γ-zein band appeared in all samples and increased in intensity for the γ-rich pellet. The γ-zein band (27 kDa) increased in intensity for all samples, except for S zein. SDS-PAGE analysis of untreated samples showed that S zein, P zein, and α-rich extract were similar in composition, except for the content of γ-zein. P zein showed the highest content of γ-zein, followed by α-rich extract and S zein, respectively. High molecular weight fractions were attributed to oligomers of α-, β-, and γ-zein of varied composition.

Rheological Behavior. G' and G'' of zein solutions at 27% (w/w) in 70% ethanol, run at 1 Hz and 0.3% strain, are shown in Figure 2, panels a, S zein, b, P zein, and c, α-rich extract. Figure 2 shows the change in G' and G'' over time, where G' (storage modulus) represents the solid-like behavior of the material and G'' (loss modulus) represents liquid-like behavior.[29] Figure 2a shows nearly constant values of G' and G'' for S zein through the duration of the experiment (4 h). In Figure 2a, G' was always lower than G'', indicating that the system was dominated by liquid-like properties. Figure 2b shows that both G' and G'' increased for P zein over time. G'' dominated the system in the first period of the experiment, indicating the liquid-like behavior of the solution. However, G' increased sharply and finally crossed over with G'' at 75 min, indicating the gelation point of the system. The gelation point is defined as the point at which the first macromolecule of infinite dimensions is formed and determines the transition from liquid to solid.[31] Tung and Dynes[32] identified the gelation point as the crossover of G' and G'' moduli in small-amplitude oscillatory shear experiments. In a typical cross-linking polymerization,[32] initially, when the sample is in in the liquid state, the viscous properties are dominant and more energy is dissipated than stored ($G'' > G'$). When polymerization is completed (solid state), the elastic properties dominate and more energy is stored than dissipated, so that $G' > G''$.

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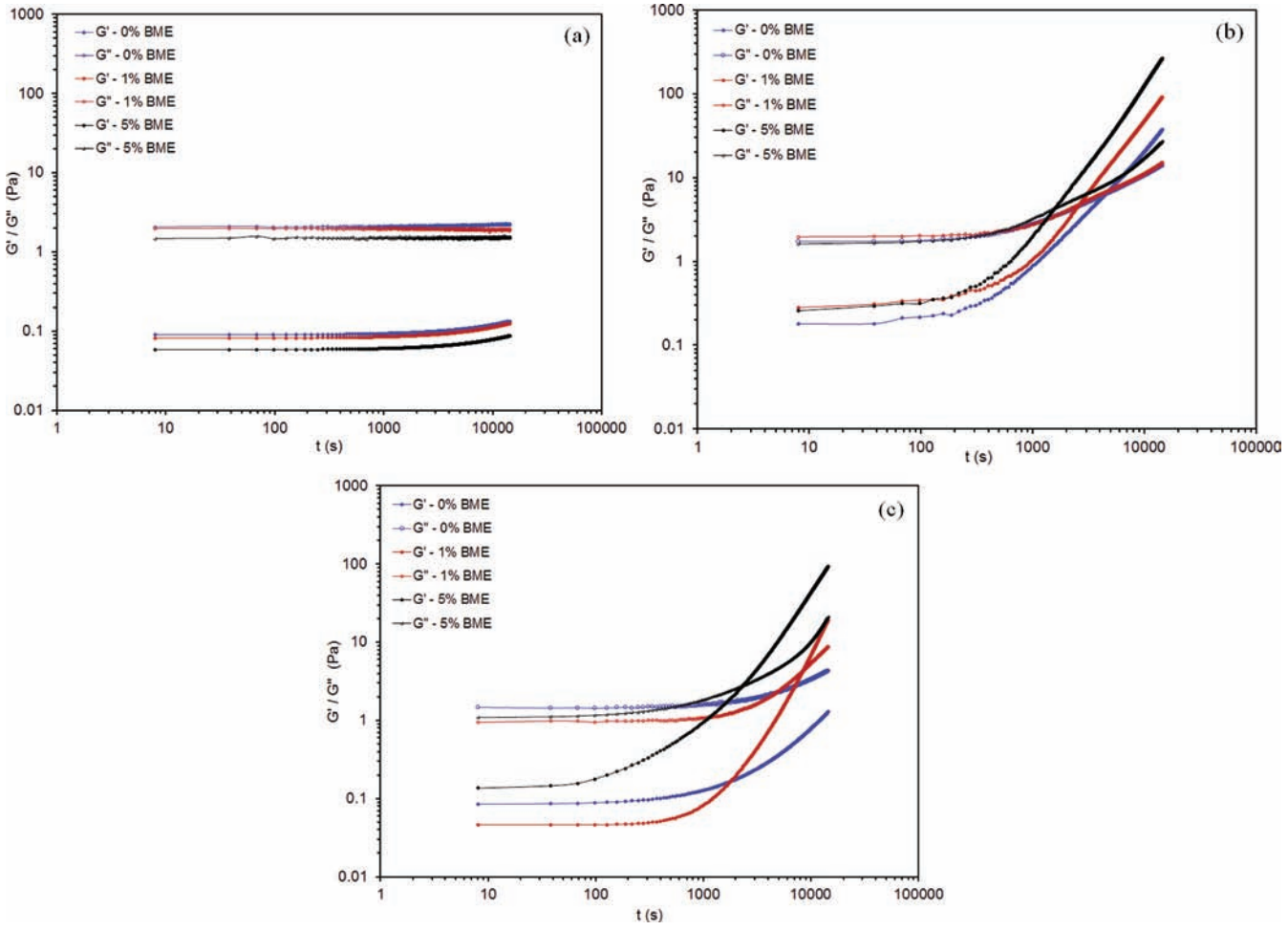

Figure 3. Oscillatory time sweep results of zein solutions, 27% (w/w) in 70% ethanol containing 0, 1, and 5% BME, run at 1 Hz and 0.3% strain: (a) S zein; (b) P zein; (c) α-rich extract.

Consequently, the point at which $G' = G''$ may be used to define the gelation point. After the crossover point, in Figure 2b, G' increased continually at a higher rate than G'', indicating that the system was more solid-like. Figure 2c shows G' and G'' of the α-rich extract. G' and G'' values were constant at the start of the experiment, but increased with longer test times. Although G' increased at a higher rate than G'' during the first 4 h of the experiment, G' was still lower than G''. This result indicated that the system was dominated by liquid-like properties. Such behavior indicated that zein extracted from whole corn with 70% ethanol (P) differed in rheological behavior from commercial α-zein (S). P zein showed a measurable gelation time. SDS-PAGE analysis (Figure 1) revealed that P zein had a higher content of γ-zein than S zein and α-rich extract, which suggested the involvement of γ-zein in the gelation processes. BME, used to reduce disulfide bonds, was added to the solvent in the next experiment to investigate the role of disulfide bonds in gelation.

Effect of BME on Rheological Behavior. Oscillatory time sweep results in Figure 3a show that BME did not affect the behavior of G' and G'' of S zein. Because the main effect of BME on proteins is the breaking of disulfide bonds and S zein contains only a minor amount of cysteine groups (capable of forming disulfide bonds), it was suggested that BME had limited interaction with S zein and that its original rheological behavior was maintained after the addition of 1 and 5% BME. On the other hand, BME decreased the gelation time for P and α-rich extract (see Table 2). For both samples, the gelation time decreased further with increasing BME concentration (Figure 3b,c). This behavior was believed to be due to the breaking of disulfide bonds, which likely involved γ-zein 16 and 27 kDa, which have high cysteine contents (see Table 1). In Figure 1, the breaking of intermolecular disulfide bonds is evidenced by the dissociation of high molecular weight zein oligomers. However, it was believed that this process did not have a major effect on gelation time because it also occurred in S zein, which was not affected by BME. It was thus suggested that the breaking of intramolecular disulfide bonds mainly in γ-

Table 2. Rheological Data of BME-Treated Samples

sample (27% w/w in 70% ethanol)	gelation time (min)	storage modulus, G' (Pa)	
		at crossover	at 4 h
P zein (0% BME)	75	6.25	37.24
P zein (1% BME)	47	5.18	86.51
P zein (5% BME)	25	4.30	263.71
α-rich extract (0% BME)	>240		1.24
α-rich extract (1% BME)	146	4.48	15.72
α-rich extract (5% BME)	38	2.58	68.51
S zein (0, 1, and 5% BME)	>240		<0.15

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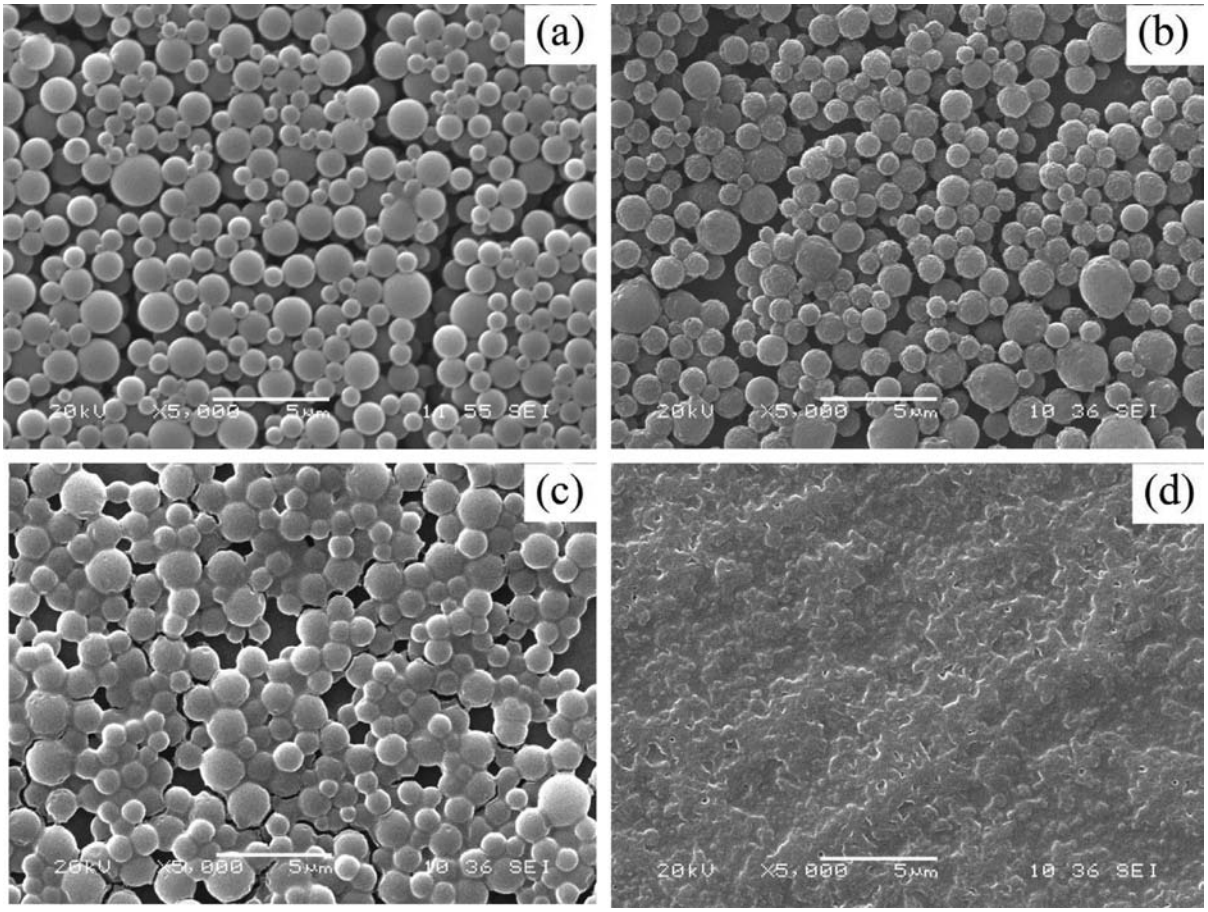

Figure 4. SEM images of zein microstructure after solvent (70% ethanol) evaporation at room conditions: (a) S zein; (b) P zein; (c) α-rich extract; (d) γ-rich pellet.

zein led to shorter gelation times. BME allowed unfolding of this peptide and the development of polymer entanglements by the formation of new hydrophobic interactions and/or hydrogen bonds. This may explain the observed decreasing gelation time with increasing BME. Havea and co-workers[33] observed a similar behavior with heat-induced whey protein gels. They measured G' of concentrated whey protein solutions with added dithiothreitol (DTT). They proposed that heating resulted in protein unfolding, which exposed hydrophobic groups and led to aggregation. Also, the breaking of disulfide bonds allowed for increased molecular movement, which possibly contributed to the rapid formation of gel networks.

Modulus was also affected by BME addition for P zein and α-rich extract (see Table 2). Moduli at the gelation point were the lowest for samples with 5% BME, followed by samples with 1% BME and no BME added. BME had a larger effect on G' than on G''. G' increased at a faster rate with higher levels of BME for P zein and α-rich extract. Higher G' values indicated the formation of stronger gels.

Microstructure. SEM images of the microstructure of P zein, S zein, α-rich extract, and γ-rich pellet are shown in Figure 4. Panels a–c of Figure 4 show the formation of α-zein microspheres, 500 nm to 2 μm in diameter. Wang and Padua[34] described the formation of zein microspheres from ethanol–water solutions and explained it in terms of evaporation-induced self-assembly processes. During drying, ethanol evaporates more rapidly than water, turning the solvent media increasingly hydrophilic. α-Zein is a relatively hydrophobic protein and tends to form hydrophobic associations in aqueous media, which drive self-assembly processes.

Figure 4d shows the continuous film structure of the γ-rich pellet. γ-Zein has a high content of cysteine residues, which are able to participate in zein-to-zein disulfide linkages, presumably responsible for the formation of networks. All zein samples (S zein, P zein, α-rich extract, and γ-rich pellet) formed continuous networks after drying, when the solvent contained 5% BME (Figure 5).

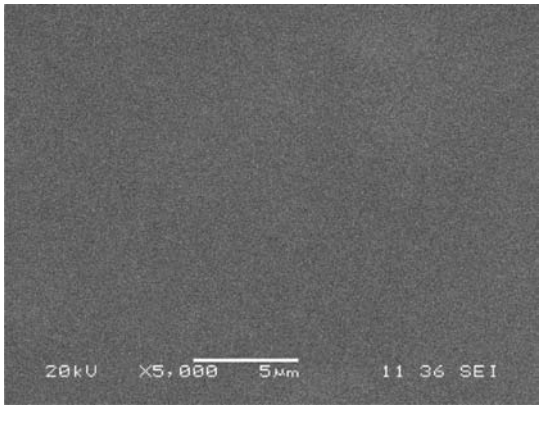

Figure 5. SEM image of S zein microstructure after solvent (70% ethanol containing 5% BME) evaporation at room conditions.

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