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Fractionation of Zein by Size Exclusion Chromatography

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Zein is a group of alcohol-soluble corn proteins, which consists of several individual proteins. A single-step gel filtration chromatography method was developed to fractionate individual zeins from ethanol extracts of whole corn. A Superdex prep 75 column was used with different mobile phases to fractionate the zeins, which were analyzed by SDS-PAGE and UV spectrophotometry. With 70% aqueous ethanol as the mobile phase, fractions containing a mixture of α-zein/*β*-zein and α-zein/*δ*-zein were obtained. With ammonium bicarbonate added to the 70% ethanol mobile phase, it was possible to obtain *β*-zein and *δ*-zein fractions devoid of other proteins. However, all fractions containing α-zein also contained minor amounts of *δ*-zein and/or *β*-zein. Almost all fractions also contained non-protein impurities.

KEYWORDS: Zein; α-zein; *β*-zein; *δ*-zein; nonideal size exclusion chromatography; mobile phase

INTRODUCTION

Zein refers to a family of alcohol-soluble proteins in corn known historically as prolamines. Prolamines in corn (maize) are known as zein, while they are known as hordein in barley, kafirin in sorghum, and gliadin in wheat (*1*). Zein has several current uses in the food industry and has potential industrial value as a biodegradable polymer, eco-friendly chewing gum, and an encapsulation agent (*2*). The individual proteins of zein were classified by Esen (*3*, *4*) on the basis of their solubility and molecular weights. There are at least four major fractions: α-zein (molecular weight of 21−25 kD), which is the most abundant fraction, *β*-zein (17−18 kD), *γ*-zein (27 kD), and *δ*-zein (9−10 kD), which is often included in α-zein because of its immunological relationship to α-zein (*3*, *4*). These fractions of zeins differ in their amino acid sequences and surface charges. In addition, individual zeins vary in composition and concentration based on corn genotypes (*4*, *5*), location of protein bodies in the kernel (*6*, *7*), and age of the kernel (*8*, *9*). High lysine varieties such as *opaque-2* and QPM (Quality Protein Maize) have a low α-zein content (*10*−*12*), and this could have an effect on the purification of individual zeins.

Currently zein is manufactured by Freeman Industries LLC, Tuckahoe, NY, in two primary forms (Freeman white and Freeman yellow zein) and by Showa Sangyo Co., Tokyo, Japan. According to our SDS-PAGE analysis (shown later in **Figure 1**), commercial zeins are mainly α-zein with small amounts of other zeins. It is used in plastics, fibers, foods, pharmaceuticals, and nutraceuticals (*2*). However, other zeins have not been used for specific applications nor extensively studied. Therefore, it would be interesting to be able to purify and investigate the properties of zeins other than α-zein. Some studies have shown

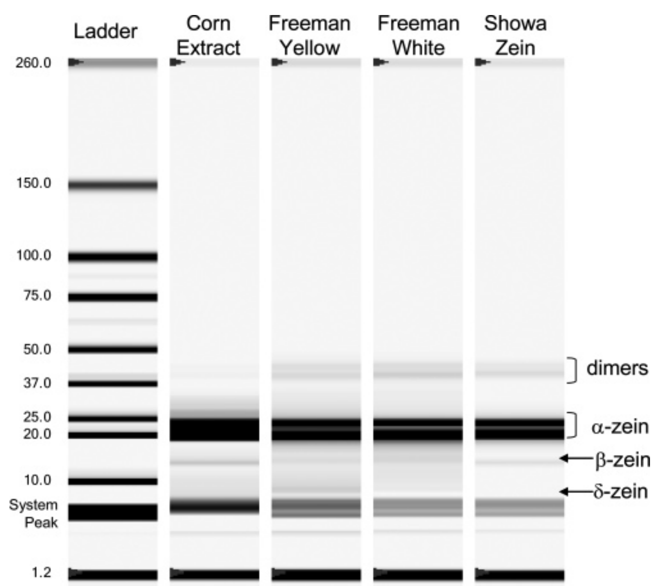

Figure 1. SDS-PAGE of corn extract and commercially available zeins.

differences in physical properties of corn proteins and individual zein fractions between corn varieties (*13*, *14*).

Because of the similarity in physicochemical properties of the individual zeins, their fractionation, separation, and purification are difficult. Zein has been analyzed and purified using various chromatographic methods. Gel filtration was used to isolate monomers and dimers of α-zein (*15*) and to decolorize zein (*16*). Ion exchange chromatography yielded multiple subfractions of α-zein (*17*, *18*); however, isoelectric focusing (IEF) of the proteins revealed some charge heterogeneity. Cabra et al. (*19*) isolated α-zein of high purity using SP-Sepharose cation exchanger, which separates by both size and charge. Reversed

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Table 1. Fractions from Size Exclusion Chromatography of Ethanolic Corn Extract Using 70% Aqueous Ethanol as the Mobile Phase[a]

group	A		B		C		D			E	F G
fraction	1	2	3	4	5	6	7	8	9	10	11
α-zein	+	+	+	+					+	+	+
β-zein			+	+					+	+	+
δ-zein	+						+	+	+	+	
impurities			+	+	+	+	+	+	+	+	

[a] Chromatogram is shown in **Figure 2**, and SDS-PAGE is shown in **Figure 3** (+ indicates that a particular zein was present in that fraction).

phase chromatography was used to separate zein fractions on the basis of their hydrophobicity. Multiple components were apparently observed by IEF in each fraction (*20*, *21*). The literature on chromatographic separation of zein indicates that sample preparation is important because different extraction conditions lead to different extract composition, which in turn affects the separation and purification.

The objective of this work was to separate individual zeins using size exclusion chromatography (SEC). The SEC gel used as the stationary phase was Superdex 75 prep grade media, which is an epichlorohydrin-cross-linked agarose−dextran complex. The effect of modifying the mobile phase on zein separation was also studied; for example, urea was added to the mobile phase to eliminate hydrophobic interactions, and ammonium bicarbonate was used to adjust the pH to suppress electrostatic interactions.

MATERIALS AND METHODS

Preparation of Corn Extract. To maximize recovery of individual zeins, raw unprocessed corn was used as our starting material instead of corn processing byproducts that are typically used commercially. The extraction procedure is based on our earlier work (*22*). Whole raw corn (yellow dent #2 containing 14−16% moisture) was obtained from Anderson Grain Co. (Champaign, IL). It was ground at room temperature with a bench top hammer mill (IKA MF 10.2, IKA Works Inc., Wilmington, NC) with 1 mm screen. Ethanol (200 proof; USP grade) was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, KY). The water was distilled and then microfiltered using a 0.2 μm Maxi Capsule Filter (Pall-Gelman, Ann Arbor, MI). The ground corn was immediately mixed with the solvent, which was 70% (v/v) aqueous ethanol that had been preheated to 50 °C, in a ratio of 4 mL of solvent to 1 g of ground corn. The mixture was stirred for 30 min at 50 °C. The suspension was filtered through Whatman #1 filter paper (11 m pore size rating) under vacuum. The clear solution was kept at 40 °C until used to minimize zein precipitation.

Chromatography. Tricorn semi-prep chromatography columns (1 cm diameter, 60 cm length, 47 mL column volume) were obtained from GE-Healthcare (Piscataway, NJ). Two of these columns were connected with a column connector to increase the column length and improve the separation. The columns were packed with Superdex prep 75 resin (GE Healthcare, Piscataway, NJ). The equipment used in these experiments was a semi-prep Waters system (600E pump, 2996 PDA detector, and Empower software from Waters Inc., Milford, MA). The absorbance of the samples was measured with the PDA detector at a wavelength of 280 nm.

Three mobile phases were studied: 70% v/v aqueous ethanol, 4 M urea/70% ethanol, and 0.2 M ammonium bicarbonate/70% ethanol at pH 8.1. The 70% ethanol was chosen as the mobile phase because the ground corn was extracted with 70% ethanol. The flow rate was 0.5 mL/min, which was the maximum recommended by the manufacturer based on the pressure limits of the column and resin. Experiments were conducted at room temperature (22 ± 1 °C). The corn extract was filtered with a 0.2 μm PTFE hydrophilic syringe filter (Millipore, Billerica, MA) prior to injection. For the experiments with the modified mobile phases, the corn extract was premixed with urea or ammonium bicarbonate at a concentration equal to that in the mobile phase before filtration. Injection size was 2 mL.

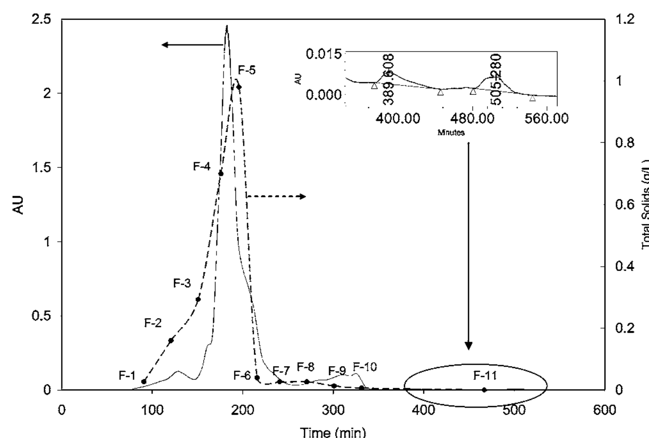

Figure 2. SEC chromatogram of corn extract with 70% ethanol mobile phase. F-1–11 are fractions collected for further analysis. The solid line represents the chromatogram at 280 nm, and the dashed line represents total solids of fractions.

Total Solids and Protein Concentration. Total solids of the samples were determined by gravimetric analysis. Samples were oven-dried at 100 °C for 12 h, cooled, and weighed. The Bio-Rad assay was adapted to measure protein concentration in the ethanol solutions. The dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA) was diluted 3× with DI water. One milliliter of sample was mixed with 4 mL of dye reagent and vortexed. The optical density at 595 nm was measured within 1 h, taking due care to prevent evaporation of reagents and samples because ethanol concentration affects the measurements. Standard curves were established using a purified commercial zein (Showa Sangyo Co., Tokyo, Japan) dissolved in the individual mobile phases.

Electrophoresis. SDS-PAGE was performed with a Bio-Rad Experion system (Bio-Rad Laboratories, Hercules, CA) or with a Mini Protean III gel electrophoresis cell (Bio-Rad Laboratories, Hercules, CA). Samples containing urea were dialyzed using regenerated cellulose (MWCO 3500, Fisher Scientific, Pittsburgh, PA) against 70% ethanol before drying. Samples were dried before electrophoresis.

When the Experion unit was used, the dried samples were dissolved in 6 μL of buffer under reducing conditions and heated at 95−100 °C for 3 min. Samples were then loaded onto a Pro260 chip for the analysis. When the Mini Protean II was used, the dried samples were dissolved in the reducing sample-loading buffer to make a 1−5 mg/mL solution (because samples were collected from a single run, lower concentrations were used only if there was not enough protein). Samples were then heated at 95 °C for 3 min and loaded onto Bio-Rad tris-HCl ready gels (12% resolving gel and 4% stacking gel) and run at 125 V for 1 h at room temperature (25 °C). The gels were then stained in Commassie Brilliant Blue R-250 solution and destained in 10% acetic acid and 40% methanol solution.

Spectral Analysis. The spectra of selected fractions were determined with a 2996 photodiode array detector (Waters, Milford, MA). The fractions were taken from the middle of the retention times of the eluting peaks.

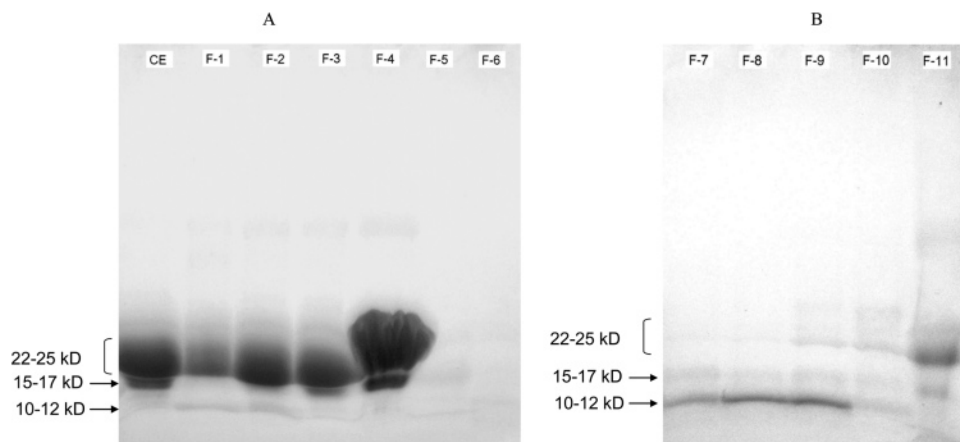

Figure 3. SDS-PAGE of corn extract and fractions obtained by SEC with 70% ethanol mobile phase. See **Figure 2** and **Table 1** for fractions. (**A**) Lane 1, corn extract (CE); lanes 2–7, fractions 1–6; (**B**) lanes 1–5, fractions 7–11.

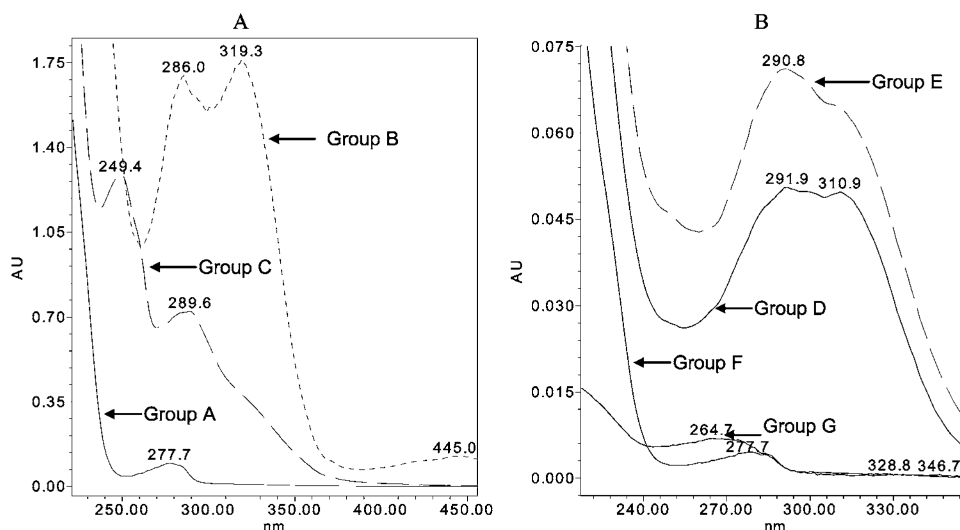

Figure 4. Spectra of the SEC fractions with 70% ethanol mobile phase shown in **Figure 2** and **Table 1**. (**A**) Solid line: spectra of 121 min, the middle point of group A. Dotted line: spectra of 176 min, the middle point of group B. Dashed line: spectra of 206 min, the middle point of group C. (**B**) Solid line: spectra of 271 min, the middle point of group D. Dashed line: spectra at 331.5 min, the middle point of group E. Dotted line: spectra at 389.6 min, group F. Dash–dotted line: spectra at 505.3 min of group G.

RESULTS AND DISCUSSION

Figure 1 is a SDS-PAGE of commercial zein products and the ethanol extract of whole corn. All of the samples show protein bands at 21−24 kD. Both Freeman products showed light bands of 9 and 17 kD, which are δ-zein and β-zein, respectively. The Showa zein showed a 17 kD β-zein band but no 9 kD δ-zein. The corn extract showed bands at 9 kD and 17 kD, although the 9 kD δ-zein band is too light to be seen in the figure.

The SEC chromatogram of the corn extract is shown in **Figure 2**. The 280 nm chromatogram showed one major peak at 188 min and two smaller peaks at 129 and 325 min. Eleven fractions were collected labeled F-1−F-11 (F-1−3, F-7−9 were 15 mL, F-4−6 were 10 mL, F-10 was 15.5 mL, and F-11 was 120 mL). The fractions were grouped together according to their major components as groups A−G (**Table 1**). Each of the 11 fractions was analyzed for total solids (also shown in **Figure 2**) and for molecular weight by SDS-PAGE (**Figure 3**). The spectra of samples collected in the midpoints of each group are shown in **Figure 4**. There were two small peaks in F-11 (see inset of **Figure 2**), and the spectra of both peaks are shown in **Figure 4B**.

Molecular Weight Distributions by SDS-PAGE. The components of each fraction are shown in **Table 1** with the major zeins of each fraction highlighted. Based on the molecular weights obtained from electrophoresis, group A (fractions 1 and 2) consisted mainly of α-zein of 21−24 kD with small amounts of δ-zein. The dominant protein in group B (F-3 and F-4) is β-zein, but it also includes a fairly high level of α-zein. The α-zein and β-zein could not be completely separated due to the similarity in their molecular weights and the limitation of the SEC resin. Column length could not be increased to improve resolution because it also increased pressure drop above recommended limits. Decreasing flow rate also was not an option because it increases the effects of longitudinal diffusion, which causes band broadening and further decreases resolution (*23*).

Interestingly, as shown in **Figure 3**, there is no protein in group C (F-5 and F-6). Fractions 7 and 8 of group D are primarily δ-zein, but fraction 9 of group D shows some α-zein and β-zein. Fraction 10 (group E) is a mixture of all of the zeins with similar concentrations. Fraction 11 contains both α-zein and β-zein. Although there is considerable overlapping of peaks, some of the zeins were purified or enriched. The

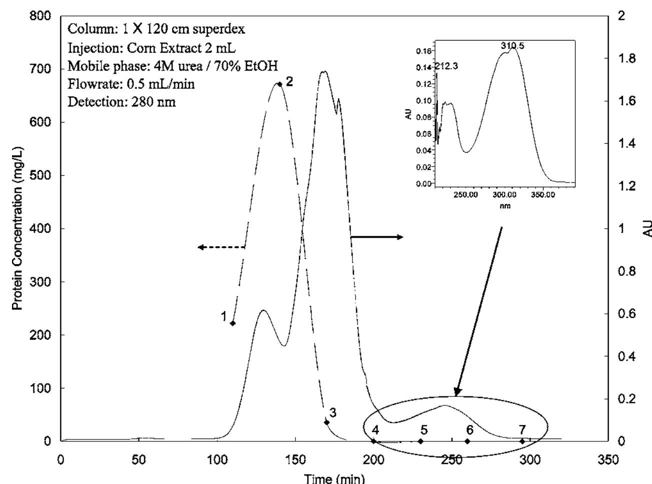

Figure 5. SEC chromatogram of corn extract with urea–ethanol mobile phase. The solid line represents the 280 nm chromatogram, and the dashed line is protein concentration of the fractions. The inset is the spectra for the third peak (fractions 4–7).

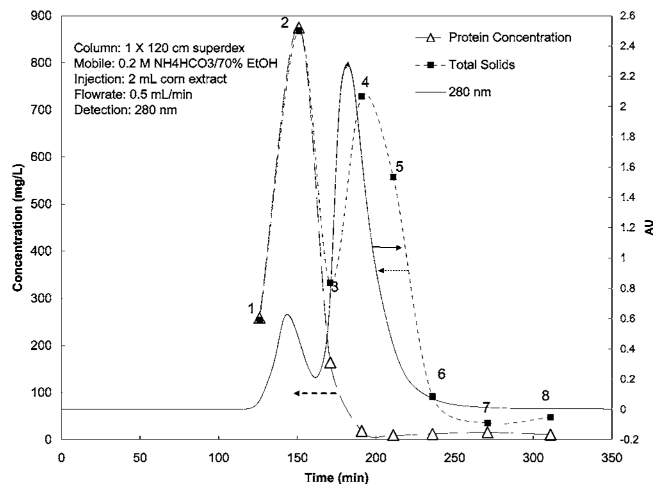

Figure 7. Chromatogram of SEC of corn extract with the ammonium bicarbonate–ethanol mobile phase. Solid line: absorbance at 280 nm. Dotted line: total solids. Dashed line: protein concentration. F-1–8 are fractions collected for further analysis.

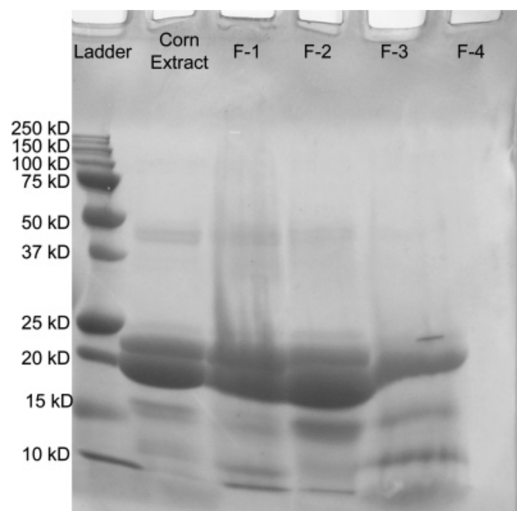

Figure 6. SDS-PAGE of corn extract and fractions of SEC with urea–ethanol mobile phase. See **Figure 5** for fractions. Lane 1: molecular weight markers. Lane 2: corn extract. Lanes 3–6: fractions 1–4.

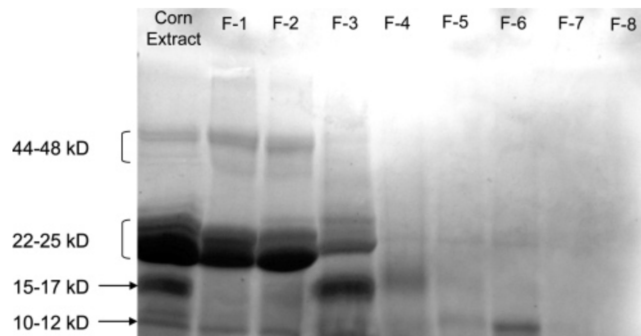

Figure 8. SDS-PAGE of zein fractions obtained from SEC of corn extract with the ammonium bicarbonate–ethanol mobile phase. See **Figure 7** and **Table 2** for fractions. Lane 1: corn extract. Lanes 2–7: fractions 1–6.

elution patterns (appearance and reappearance of the same protein during elution) and timing of the various fractions indicate that adsorption effects are affecting the separation. Similar adsorption effects were observed by Drevin et al. (*24*), as discussed later.

Spectral Analysis. The molecular weight data were confirmed with a spectral analysis of the fractions (**Figure 4**). Group A is zein because the spectra are identical to that of pure industrial zein (*25*) (maximum of 277.7 nm, **Figure 4A**, solid line). The spectra of group B (**Figure 4A**, dotted line) show maxima at 286 and 319 nm. Group C shows impurities at 249 and 289 nm (**Figure 3A**, dashed) but this group showed no protein band in SDS-PAGE (**Figure 3**). Proteins reach the maximum in F-4, while impurities reach the maximum in F-5.

The spectra of group D (**Figure 4B**, solid line) showed maxima at 292 and 311 nm, which resemble the spectra of the impurities fractions. However, F-7 and F-8 also contain δ-zein. Group E (**Figure 4B**, dashed line) has spectra similar to those of group D but at different intensities. Group E is a mixture of all proteins and the impurities. Fraction 11 had two peaks (**Figure 2**) with different spectra (shown in **Figure 4B**, group F and group G).

Impurities. The spectral and PAGE analyses indicate there are non-protein UV-active substances in the extract that sometimes mask the protein peaks in the chromatograms. These are referred to as "impurities" in this paper. It is unlikely that these are oil or lipids because 70% aqueous ethanol does not extract oil (*26*). Based on visual inspection, the solids in fractions 4−6 are highly hygroscopic, and the spectra of F-4 (group B, dotted line in **Figure 4A**) show two peaks at 286 and 319 nm. The impurities could be polyamine conjugates, which have spectra similar to that of F-4. Ethanol extracts of corn bran and corn fiber contain *p*-coumaroyl-feruloyputrescine (CFP, MW 410) and diferuloylputrescine (DFP, MW 440), yielding spectra with a maximum of ∼320 nm and another peak at ∼285 nm (*27*, *28*). It is interesting that, although the concentrations of CFP and DFP are not high, they yielded a very high absorbance of 1.7 AU in this work.

The spectra of group C were similar to those of group B (**Figure 4A**) with the first peak at 290 nm and a second peak of lower intensity. The spectra of groups D and E (**Figure 4B**) were also similar but with a lower intensity. Polyamine conjugates elute with the proteins in these fractions. Fraction 11 appears to be proteins without polyamine conjugates, based on the spectra of groups F and G (**Figure 4B**).

Adsorption Effects. The volume of the empty column is 94 mL, and the volume occupied by the Superdex 75 matrix is 20% of column volume (*29*). Therefore, a completely included component should elute before 75 mL, equivalent to a retention

Table 2. Fractions from Size Exclusion Chromatography of Ethanolic Corn Extract Using Ammonium Carbonate–70% Aqueous Ethanol as the Mobile Phase[a]

group	A		B	C	D		E	
fraction	1	2	3	4	5	6	7	8
α-zein	+	+	+					
β-zein			+	+				
δ-zein	+	+	+		+	+		
impurities			+	+	+	+	+	+

[a] Chromatogram is shown in **Figure 7**, and SDS-PAGE is shown in **Figure 8** (+ indicates that a particular zein was present in that fraction).

time of 150 min at a flow rate of 0.5 mL/min. All fractions eluting after 150 min (all fractions eluting after F-2) are held back due to interactions between the solute and the stationary phase. In addition, all of the zeins appear more than once in the eluted fractions, which again is an indication of interactions, probably due to adsorption. Although Superdex is a composite matrix of dextran cross-linked agarose without any functional groups, it is likely that steric hindrance is not the sole determinant of separation. Gel−solute interactions due to hydrophobic interactions, hydrogen bonding, and ionic interactions may also be important. Partitioning of solutes is also influenced by the relative polarities of the gel, solute, and the mobile phase (*30*). According to Drevin (*24*) and Hellberg (*31*), at low ionic strength, electrostatic interactions dominate. When the pH of the system is less than their pI, proteins are positively charged and bind to the negatively charged resin. When pH > pI, proteins are negatively charged, and ion exclusion occurs. On the other hand, when the ionic strength is high, hydrophobic interactions dominate (*24*, *32*, *33*). The Superdex resin contains about 1 μmol/mL gel of negatively charged carboxyl groups, and the extent of hydrophobic interactions is determined by the amount of dextran (*31*). A similar combination of ionic and hydrophobic interactions occurs with Superose (a cross-linked agarose); in fact, virtually all current known gel filtration media are more or less hydrophobic and are weak action exchangers (*34*).

Modifying Solute−Resin Interactions. The interactions between solutes and the stationary phase can be changed by adjusting the composition of the mobile phase. In this work, the mobile phases were modified by adding urea (to counter hydrophobic interactions) or ammonium bicarbonate (to increase the pH and minimize ionic interactions). The stationary phase, column dimensions, flow rate, and temperature were the same as in the earlier experiments.

Urea−Ethanol Mobile Phase. Urea is a denaturing reagent that unfolds proteins causing them to expand, resulting in larger Stokes radii than native proteins (*35*). Urea interacts preferentially with hydrophobic regions of proteins and hence suppresses hydrophobic interactions. It also interacts with the hydrophobic sites of the stationary phase and blocks interactions between solute and matrix. Urea has been used at high concentrations in mobile phases to disrupt hydrogen bonds and hydrophobic adsorption in size exclusion chromatography (*36*).

Figure 5 is a chromatogram of the corn extract with the urea−ethanol mobile phase. Seven fractions of 15 mL each were collected and analyzed for protein concentration. The elution profile is similar to the 70% ethanol mobile phase shown earlier in **Figure 2**, although the protein distributions over the fractions are different. Protein was measured only in the first three fractions. This was confirmed by the SDS-PAGE patterns of the fractions (**Figure 6**), which show protein in only the first three fractions. All individual zeins (α, β, and δ) are present in the three fractions, but in different proportions. Addition of urea to the mobile phase minimizes differences in secondary and tertiary structure of the individual zeins. However, size differences between the zeins would not be large enough for them to be separated by size exclusion alone. The presence of urea prevents hydrophobic adsorption effects as observed in **Figure 5** (fractions 4−7 showed no protein). On the other hand, these peaks contained polyamines, which eluted after one column volume, implying that electrostatic interactions between polyamines and the stationary phase are occurring.

Ammonium Bicarbonate−Ethanol Mobile Phase. Figure 7 is a chromatogram of corn extract with ammonium bicarbonate in the mobile phase. Eight fractions of 15 mL each were collected and analyzed for protein and total solids concentrations. **Figure 7** shows three parameters: total solids, protein concentration, and UV absorbance at 280 nm. The 280 nm plot (solid line) shows two major peaks, which elute together with the total solids. Because the concentrations of protein and total solids are the same, the first two fractions (F-1 and F-2) are pure protein. As shown by the SDS-PAGE of this run (**Figure 8**), these fractions consist of α-zein with a minor amount of δ-zein. This is similar to that obtained with the 70% ethanol mobile phase.

Table 2 shows the distribution of the individual zeins and impurities among the various fractions, and **Figure 9** shows their UV spectral analysis. Group A contains no impurities, while groups B−E contain non-protein impurities. Group B (F-3) contains all three individual zeins, but appears to be enriched in β-zein (**Figure 8**). Although the protein content of F-4 is only 2.5% (dry basis), this fraction is mainly β-zein and impurities. Fractions 5 and 6 consist of only δ-zein and impurities but at low protein concentrations (1.6% and 13.3%, dry basis, respectively). The protein contents of F-7 and F-8 are too low to be seen in SDS-PAGE. Thus, with this mobile phase, all α-zein elutes at or before the void volume of the column, indicating little or no interaction between α-zein and the stationary phase. On the other hand, elution volumes of β-zein and some of the δ-zein are larger than the total column volume, suggesting interactions between these zeins and the stationary phase. Some δ-zein elutes together with α-zein, and this could be because some of the δ-zein aggregates or it is negatively charged and partially excluded from some of the pores.

pH is a critical factor that affects partitioning of solutes by its influence on their net charge. Because the pI values of most of the zeins are 7−8 (*37*), the ammonium bicarbonate (pH 8.1) makes most proteins negatively charged. As the stationary phase contains negatively charged groups (*31*), the addition of ammonium bicarbonate diminishes attractive forces between the proteins and stationary phase. Thus, those fractions eluting after 188 min indicate adsorption by hydrophobic interactions rather than by ionic interactions.

Conclusions. Zeins can be fractionated by gel filtration chromatography with Superdex resin. The major protein α-zein

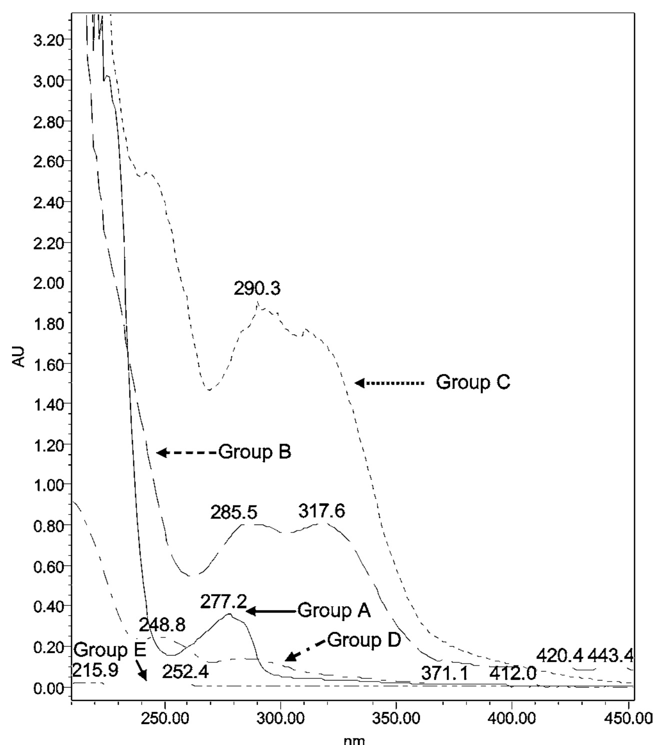

Figure 9. UV spectra of the fractions with ammonium bicarbonate–ethanol as mobile phase shown in **Figure 7** and **Table 2**. Solid line: spectra of 136 min, the middle point of group A. Dashed line: spectra of 171 min, the middle point of group B. Dotted line: spectra of 191 min, the middle point of group C. Dashed–dotted line: spectra of 226 min, the middle point of group D. Dash–dot–dotted line: spectra of 291 min, the middle point of group E.

can be eluted in one column volume with little or no impurities but with a minor amount of δ-zein associated with it. β-zein and δ-zein could be separated from other proteins, but the fractions contained non-protein impurities. Interactions between the proteins and the stationary phase could be enhanced or diminished by modifying the mobile phase, which improves the separation of proteins with similar molecular weights.

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