Separation of Alcohol-Soluble Proteins (Zeins) from Maize into Three Fractions by Differential Solubility

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ABSTRACT

The prolamin of maize (Zea mays L.), zein, was extracted from endosperm meal with 60% (v/v) 2-propanol/1% (v/v) 2-mercaptoethanol either directly or subsequent to extraction with 90% (v/v) 2-propanol. The zein extracted with 90% 2-propanol was essentially made up of 20 to 24 kilodalton polypeptides (α-zein) while that extractable with 60% 2-propanol/1% 2-mercaptoethanol contained, in addition to α-zein, 17 to 18 kilodalton methionine-rich polypeptides and a 27 kilodalton proline-rich polypeptide. While zein was separated into three fractions by differential solubility in 90% 2-propanol and 30% 2-propanol/30 millimolar sodium acetate (pH 6) using two different fractionation protocols. Each of the three solubility fractions (SF1, SF2, and SF3) had a unique polypeptide composition. Based on results obtained from two inbreds, K55 and W64A, the SF1 constituted 75 to 80% of the total zein and included as major components 20 to 24 kilodalton polypeptides and a minor 10 kilodalton polypeptide. The SF2 made up 10 to 15% of the total zein and included exclusively 17 to 18 kD methionine-rich polypeptides. A 27 kilodalton proline-rich component constituted the SF3 and contributed 5 to 10% to total zein.

Osborne (14) coined the term prolamin to refer to the alcohol-soluble proteins that occur in cereal grains. The term literally meant, in Osborne’s usage, a protein that is rich in proline and glutamine and/or asparagine. The prolamin of maize, called zein, like those of other cereals fully meets the criteria implied in Osborne’s definition. The extraction and purification of zein have been the subject of many investigations since its discovery by Gorham in 1822. Osborne (13) and Osborne and Mendel (15) performed the first systematic study of solvents and conditions for extraction of zein; they concluded that it was soluble in relatively strong alcohol, acetic acid, phenol, and dilute alkali solutions. Since then the most commonly used zein solvents have been 60 to 70% solutions of either ethanol or 2-propanol.

Zein is not a homogeneous protein species; rather it is a mixture of several groups of proteins with similar solubility behavior. Moureaux and Landry (10) and Paulis et al. (16) independently discovered the presence of an additional protein fraction extractable with alcohol if a reducing agent is included in the medium. This fraction is referred to as glutenin(17), alcohol-soluble reduced glutenin (17), zein-2 (22), and zein-like (12). Amino acid analysis data showed that the fraction extracted with alcohol under reducing conditions had the same predominant amino acids as zein but it had higher amounts of histidine, arginine, proline, glycine and methionine and lower amounts of leucine, isoleucine, aspartic acid, and phenylalanine than zein.

Separation of zein into three distinct fractions by adding water stepwise to solutions in alcohol or methyl cellosolve was reported by Watson et al. (24) and Gortner and MacDonald (5). However, Scallet (21) showed that the three fractions were not homogeneous when analyzed by moving boundary electrophoresis. McKinney (11) isolated zein from maize gluten as two separate solubility fractions: α-zein, soluble in 95% ethanol and constituting 80% of total zein, and β-zein, soluble in 60% ethanol but not in 95% ethanol. The occurrence of two such distinct solubility fractions was later confirmed by Turner et al. (23) and others. Based on electrophoretic analysis of whole, α-, and β-zein fractions with and without reduction and alkylation, Turner et al. (23) concluded β-zein was a disulfide-linked aggregate of components present in native zein and thus did not contain components unique to it. Paulis (20) extracted the native zein with 70% ethanol/0.5% sodium acetate and α- and β-zeins according to the procedure of Turner et al. (23) from maize endosperm meal. Extractability, solubility, amino acid and electrophoretic analyses showed that α-zein constituted 35% of total zein, and included two prominent bands with mol wt 22 and 24 kD, respectively, and had an amino acid and polypeptide composition similar to that of whole zein (20). As for β-zein, it failed to enter polyacrylamide gel without reduction but, after reduction, entered the gel and displayed three predominant size components with mol wts of 24, 22, and 14 kD. It also contained more histidine, arginine, proline, and methionine than did α-zein which Paulis (20) attributed to the presence of the 14 kD component in β-zein. Alcohol-soluble reduced glutenin, or zein-2, was separated into two subfractions, water-soluble and water-insoluble, by dialysis against water (8, 18). The same fraction was also separated into five subfractions by ion-exchange chromatography (2). Two of these subfractions, 4 and 5, and a protein isolated by Wilson et al. (25), reduced-soluble protein, are now known to be the same as the water-soluble alcohol-soluble reduced glutenin isolated by Paulis and Wall (18).

A simple fractionation scheme to permit separation of zein into fractions of unique polypeptide composition by differential solubility has been a subject of continuous investigation for the author in the last 7 years or so. After countless, mostly unsuccessful, experiments and years of painstaking effort such a procedure has been developed. This procedure, which separates the maize prolamin, zein, into three fractions each with unique polypeptide composition, is described and discussed in this report.

MATERIALS AND METHODS

Preparation of Cornmeal. Sources of maize seeds used for zein isolation were inbreds K55 and W64A. Endosperm meal was prepared from kernels whose germ and pericarp had been removed after soaking in water for 30 to 60 min. Endosperms were ground first in a mill and then by pestle in a mortar to pass a 150 μm sieve. The corn meals so prepared were stored in a freezer with or without prior defatting with hexanes.

Extraction of Zein. Both defatted and nondefatted meals were
used for zein extraction. Meals were extracted using three different protocols. Protocol 1 used, sequentially, as solvents 90% (v/v) 2-PrOH and 60% (v/v) 2-PrOH/1% (v/v) 2-ME. In Protocol 2 three solvents, 90% 2-PrOH, 60% 2-PrOH, and 60% 2-PrOH/1% 2-ME, were used in sequence. Ex extractions with 90% 2-PrOH and 60% 2-PrOH with or without 2-ME were repeated 5 and 3 times, respectively, in protocols 1 and 2. Protocol 3 involved the use of 60% 2-PrOH/1% 2-ME as the sole solvent to extract all of alcohol-soluble proteins and extraction was repeated 4 times. All extractions were performed at room temperature for 2 to 18 h each using 2 g endosperm meal in 30 ml Corex tubes with occasional 5 to 10 s mixing on a Vortex mixer. The solvent (ml) to meal weight (g) ratio was 10:1 and centrifugations were at 12,000g (4°C) for 10 min. Supernatants from successive extractions with the same solvent were assayed for protein by a semi-quantitative dye-binding procedure (1) and those that contained protein at or above 0.1 mg/ml were pooled and stored in tightly capped Erlenmeyer flasks at room temperature until use. Various fractions obtained with protocols 1 and 2 are identified with their protocol number and solvent, e.g. protocol 1—90% 2-PrOH-soluble, protocol 1—90% 2-PrOH/1% 2-ME-soluble, etc. The fraction obtained by using protocol 3 is designated as whole zein (WZ).

Fractionation of Zein by Differential Solubility. Separation of zein into fractions having unique polypeptide composition was investigated by exploiting possible solubility differences among constituent polypeptides at different alcohol, urea, salt concentrations, and pH values. Of numerous protocols used, two were judged to provide the most satisfactory fractionation. These protocols, referred to as A and B, respectively, were presented in the form of a flowsheet diagram in Figure 1. Both protocols are devised to fractionate zein extracted with 60% 2-PrOH or 60% 2-PrOH/1% 2-ME. If 2-ME was not included in solvent during extraction, it was added later to the extract. Protocol A involves adding 3 volumes of 2-PrOH (alcohol content is about 100%) to the zein extract or solubilized with 60% 2-PrOH to raise 2-PrOH concentration to 90% (v/v). The resulting solution was left standing overnight (16–20 h) at 4°C and then centrifuged at 12,000g (4°C) for 10 min. Two volumes of H2O were added stepwise to the supernatant, referred to as solubility fraction 1 (ASF1), to make it 30% 2-PrOH. In addition, 0.01 volume of 3 M NaAc (pH 6.0) was added to make the solution 30 mM NaAc/30% PrOH final concentrations. The solution was left standing overnight or longer at 4°C prior to centrifugation at 5,000g. The supernatant was discarded; the pellet was washed with water, dissolved in 60% tert-butanol, and freeze-dried. The pellet from 12,000g centrifugation was washed twice with 90% 2-PrOH/0.5% 2-ME, 10 to 15 min each, followed by centrifugation at 5,000g for 5 min. Then the pellet was solubilized in 60% 2-PrOH/2% 2-ME for several hours to overnight at room temperature. The solution was made first 30% 2-PrOH by adding 1 volume of water stepwise and then 30 mM NaAc as described above. The resulting 30% 2-PrOH/30 mM NaAc/1% 2-ME solution was allowed to stand at 4°C overnight and centrifuged at 5,000g for 10 min. The supernatant, designated ASF2, was reduced to about 1/2 volume by evaporation in a dialysis bag, dialyzed against H2O, and freeze-dried. The pellet, designated ASF3, was washed twice with 30% 2-PrOH/30 mM NaAc (pH 6.0/5.5% 2-ME) 10 to 15 min. It was then suspended in 60% tert-butanol and freeze-dried.

Fractionation according to the protocol B was performed as follows. Zein extracted or solubilized with 60% 2-PrOH/1% 2-ME was mixed with 1 volume of water to make it 30% 2-PrOH/0.5% 2-ME. Then, 0.02 volume of 3 M NaAc was added to 30

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Fig. 1. Flowsheet diagram showing the fractionation protocols (A and B) used to separate the maize prolamin, zein, into three fractions based on differential solubility at various alcohol (2-propanol) and sodium acetate concentrations.

mm final concentration and the solution was kept at 4°C overnight prior to centrifugation at 5,000g for 10 min. The supernatant, designated BSF3, was concentrated, dialyzed, and freeze-dried as mentioned above. The pellet was washed twice with 30% 2-PrOH/30 mM NaAc/0.5% 2-ME, followed each time by centrifugation at 5,000g. Then the pellet was solubilized in 60% 2-PrOH/1% 2-ME at room temperature for several h to overnight. Three volumes of 2-PrOH were added slowly to 90% final concentration. The solution was left standing at 4°C for overnite or longer and centrifuged at 5,000g. The pellet, designated BSF2, was washed with 90% 2-PrOH twice, dissolved in 60% tert-butanol, and freeze-dried. The supernatant, designated BSF1, was mixed with 2 volumes of water to which 3 M NaAc was added to 30 mM final concentration. The resulting cloudy solution was left at 4°C for several h to overnight and centrifuged at 8,000g. The supernatant was discarded; the pellet was rinsed with water, and then solubilized in 60% tert-butanol and freeze dried.

Analytical Procedures. All zein extracts and their fractions obtained by differential solubility using protocols A and B were analyzed by SDS-PAGE (6) and IEF (4). SDS-PAGE was performed using both homogeneous (12%, w/v) and gradient (10–15%, w/v) gels. IEF analysis was performed in 5% polyacrylamide gels containing 6 M urea and 2% (w/v) pH 3 to 10 Servalyte. Cross-linkage was 3% C in both SDS-PAGE and IEF gels. The amount of protein applied to sample slots was 25 μg for SDS-PAGE and 125 μg for IEF. The amount of protein in different solubility fractions were determined gravimetrically by weighing the freeze-dried protein powders.

RESULTS AND DISCUSSION

Zein Extraction Procedures. Of the three different extraction protocols and solvents used, 60% 2-PrOH/1% 2-ME was found to be the most effective solvent in extracting zein whether it was used as the first solvent or as 90% 2-PrOH. Extraction with neither 90% 2-PrOH nor 60% 2-PrOH was complete even when it was repeated as many as 5 or more times. In one

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1 Abbreviations: 2-PrOH, 2-Propanol; 2-ME, 2-mercaptoethanol; SF, solubility fraction; NaAc, sodium acetate; IEF, isoelectric focusing.

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experiment, extraction with 90% 2-ProOH was repeated 10 times; and all 10 extracts contained protein detectable by the Coomassie blue dye-binding method. However, the amount of protein extracted gradually decreased after each extraction. Similar results were obtained with 60% 2-ProOH whether it was used directly or after 90% 2-ProOH. In contrast, extraction with 60% 2-ProOH/1% 2-ME was essentially complete after three extractions; there was no protein detectable in subsequent extracts. These results suggest that some zein polypeptides, especially proline-rich zein (SF3) and methionine-rich zeins (SF2), occur as large homo or hetero-oligomers and multimers linked through intermolecular disulfide bonds. Therefore, reduction of disulfide bond would facilitate extractability and solubility.

As for the polypeptide composition of various extracts, the protein extracted with 90% 2-ProOH was exclusively made up of 20 to 24 kD zein polypeptides and a minor 10 kD polypeptide. This fraction corresponds to the one McKinney (11) referred to as α-zein. Protein extracted with 60% 2-ProOH included 17 kD and 18 kD methionine-rich polypeptides in addition to α-zein polypeptides. If meal was extracted directly with 60% 2-ProOH, α-zein polypeptides constituted nearly 90% of the protein. When extraction with 60% 2-ProOH was preceded by extraction with 90% 2-ProOH, α-zein polypeptides constituted about 50% of the protein in 60% 2-ProOH extracts. Protein extracted with 60% 2-ProOH/2-ME subsequent to either 90% or 60% 2-ProOH extractions, or both, contained as predominant components the 27 kD proline-rich zein (30–35%) and 17 and 18 kD (together 40 to 45%) methionine-rich zeins (3, 18, 19). In addition, it contained the α-zein polypeptides as minor components. These values are from inbred K55 and expected to vary with genotype. When meal was extracted directly with 60% 2-ProOH/2-ME, the extract contained all alcohol-soluble proteins, 20 to 24 kD zeins being predominant polypeptides (about 80%) followed by 17, 27, 18 and 10 kD ones. The 27 kD polypeptide was not readily visible in the whole zein SDS-PAGE profile of inbred W64A while it was easily detectable in the whole zein profile of K55 (Fig. 2b, lane 1). Although the amount of protein extracted with each solvent in a given extraction protocol was not quantitatively determined, in an earlier study (3) it was found that 65% of the total alcohol-soluble nitrogen was extracted from W64A with 90% 2-ProOH. The remaining 35% was extracted with 60% 2-ProOH/1% 2-ME.

Electrophoretic analysis of extracts clearly showed all zeins but the 27 kD zein were extractable with 60% 2-ProOH. Protein extractable with 90% 2-ProOH is unique in that it contains exclusively α-zeins and thus this solvent permits isolation of α-zeins free of other zeins. However α-zeins occur as a major contaminant in fractions extracted with both 60% 2-ProOH and 60% 2-ProOH/2-ME later. As for 17 and 18 kD methionine-rich zeins, they showed limited extractability with 60% 2-ProOH but were readily extractable when 2-ME was included in the same solvent. The 27 kD proline-rich zein occurred only in extracts made with 60% 2-ProOH/2-ME indicating that this protein was probably a part of a large multimeric complex held by disulfide bonds. Wang and Esen (unpublished data) showed that the 27 kD zein occurred in protein bodies along with other zeins. Ludevid et al. (9) also localized the 27 kD zein in protein bodies by immunocytochemical procedures. These results show that extraction of zein sequentially with alcohol and alcohol containing a reducing agent does not yield fractions with distinct polypeptide composition. Therefore, fractions designated zein-1 and zein-2, or zein and glutelin-1, or zein and alcohol soluble glutelin contain the same polypeptides (except 27 kD which is extracted only upon reduction) but at different relative proportions.

**Separation of Zein into Three Fractions by Differential Solubility.** Both protocol A and B separated the total alcohol-soluble protein (whole zein) into three fractions, each fraction having a distinct polypeptide composition (Fig. 2). The results which formed the basis for arriving at the two fractionation protocols were as follows. (a) α-zein polypeptides (20–24 and 10 kD zeins) are soluble in 40 to 95% 2-ProOH while 17 and 18 kD zeins are soluble in the range of 30 to 80% 2-ProOH and 27 kD zein in 0

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**Fig. 2.** Polypeptide composition of the three zein fractions isolated by separation based on differential solubility. Whole zein (zein extracted directly with 60% [v/v] 2-ProOH/15% [v/v] 2-ME) and its three solubility fractions were subjected to SDS-PAGE in 10 to 15% (w/v) polyacrylamide gradient (a) and 12% (w/v) homogenous gels (b). The first lane in both gels contained mol wt standards (BSA, 67 kD; egg albumin, 43 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 20.1 kD; myoglobin, 16.0 kD; lysozyme, 14.3 kD; Cyt c, 12.5 kD; aprotinin, 6.2 kD). Lanes 1 to 7 and 8 to 14, whole zein and its fractions from inbreds K55 and W64A, respectively. Lanes 1 and 8, whole zein; lanes 2 to 4 and 9 to 11 show profiles of SF1, SF2, and SF3 obtained with protocol A; and lanes 5 to 7 and 12 to 14 show profiles of the corresponding fractions obtained with protocol B. Note that polyacrylamide gradient (a) resolves polypeptides in SF2 better, in which two different size species within 17 kD zein are evident, than homogeneous (b) gels. In contrast, homogeneous gel resolves the α-zein polypeptides (SF1) better where three different size species within 24 kD component (also known as 21 kD zein) are easily visible. Also note a group of three bands (arrow) between 25 and 30 kD region in SF2 of inbred K55 (lanes 3 and 6).
to 80% 2-PrOH under reducing conditions. This information was effectively exploited to separate α-zeins from other zeins (17, 18, and 27 kD) by adding pure 2-PrOH to 60% 2-PrOH/2% 2-ME extracts to 90% final concentration. (b) α-zeins and 17 and 18 kD zeins precipitate completely in 30% 2-PrOH/30 mM NaAc (pH 6.0) while 27 kD zein remains in solution. This information was used to separate 27 kD zein from other zeins by adding first 1 volume of water to 60% 2-PrOH/1% 2-ME extracts and then 3 mM NaAc (pH 6.0) to 30 mM final concentration. Protocols A and B incorporates both of these principles but uses them in opposite sequence. For example, protocol A, as a first step, exploits the solubility of α-zeins and insolubility of 17, 18, and 27 kD zeins in 90% 2-PrOH to separate them from each other. In the second stage, 27 kD zein is separated from 17 and 18 kD zeins by exploiting their solubility differences in 30% 2-PrOH/0.5% 2-ME/30 mM NaAc (pH 6.0). Protocol B first separates 27 kD zein from others and then α-zeins are separated from 17 and 18 kD zeins.

One could predict from the above-mentioned results that fractions ASF1 and BSF1 would contain α-zein polypeptides when analyzed by SDS-PAGE. This expected result was indeed realized as shown in Figure 2, lane 2, which has the profile ASF1. The profile includes the major 20 to 24 kD α-zeins and the minor 10 kD α-zein. This profile is also essentially identical with that of authentic α-zein directly extracted from meal with 90% 2-PrOH. Similarly, fractions ASF2 and BSF2 would consist of 17 kD and 18 kD methionine-rich zeins and the profile of ASF2 shown in Figure 2, lanes 3, 6, 10, and 13 confirms this expectation. Also, a group of three bands (25–26 kD) consistently appeared in the profile of fractions ASF3 and BSF3 (Fig. 2, lanes 3 and 6, arrow) from inbred K55. This triplet occurs in a genotypic-specific manner in maize inbreds and has not been reported before; its relevance to other zeins is presently not known. Finally, fractions ASF3 and BSF3 would be expected to have only the 27 kD proline-rich zein; this expectation, too, is realized as can be seen in Figure 2, lanes 4, 7, 11, and 14. Moreover, differential solubility protocols A and B did not lead to the loss of any of polypeptides present in the starting material (parent fraction). When 60% 2-PrOH/1% 2-ME-soluble (protocol 1) fraction and its solubility fractions (ASF1, ASF2, and ASF3) were subjected to IEF (not shown) they all exhibited extensive charge heterogeneity. The extent of charge heterogeneity was similar in all three solubility fractions, each having about 15 charge components. Moreover, most charge components in each profile occupied distinct positions along the pH gradient indicating their being unique to their respective solubility fractions.

Comments on Fractionation Protocols. These results clearly show that two differential solubility protocols used separate zein into three fractions with distinct polypeptide composition. Both protocols are rather simple to follow and fractionation can be stopped at a convenient point and continued later. They are also amenable to scaling up. It was found that both α-zein and methionine-rich zeins could actually be salted out in the presence of NaAc at molarities less than 30 mM in 30% 2-PrOH. In a systematic study, 2.5 mM NaAc was found to be sufficient to completely precipitate α-zein in 30% 2-PrOH upon centrifugation. In contrast, proline-rich zein did not salt out with either NaAc or (NH4)2SO4 even at 1 M final concentration in 30% 2-PrOH. Although a thorough study of the effect of pH was not carried out, pilot experiments suggested that the pH optimum for salting-out of α- and methionine-rich zeins was about 6. Moreover, pellets containing 17 and 18 kD zeins were not readily soluble in 60% 2-PrOH/1% 2-ME especially when protocol B was used. This problem was solved by soaking the pellet in solvent for several hours and then heating at 50°C for 10 min. It was also observed that the solubility of 17, 18, and 27 kD zeins decreased as the number of manipulations (e.g., salting out, dialysis, freeze-drying) they were subjected to increased. Apparently exposure of these proteins to salts and aqueous media lead to new associations and conformational changes that reduce their solubility.

Washing of pellets after centrifugation with the medium in which they were precipitated was a necessary step to remove contaminating polypeptides left from the supernatant. Washes were also important to solubilize contaminants present in the pellet.

Relative Contribution of Three Solubility Fractions to Total Zein. One often finds in the literature stated that zein constitutes 50 to 60% of the total endosperm protein. However, there are no data on the relative contribution of individual zein polypeptides and size classes to the total zein. To estimate the relative contribution of three solubility fractions to total zein, whole zein extracts (protocol 3) having approximately 2 mg/ml protein were separated into three fractions according to protocols A and B. Results showed that α-zein (SF1) makes up about 81% of total zein followed by 17 and 18 kD methionine-rich zeins (SF2) (13–15%) and 27 kD proline-rich zein (SF3) (5–6%) in inbred W64A. Corresponding percentages for inbred K55 were 76 to 79%, 11 to 15%, and 9 to 10%, respectively. Thus it is clear that α-zein (SF1) is the predominant zein making up about 75 to 80% of the total zein and this percentage seems to differ very little from inbred to inbred. However, both 17 and 18 kD zeins (SF2) and 27 kD zeins (SF3) were found to vary among 10 inbreds studied (not shown) with respect to their percentage within total zein. The proportions of the three solubility fractions were also estimated in 60% 2-PrOH/2-ME-soluble fraction extracted according to the protocol 2. In this case, percentages of SF1, SF2, and SF3 were, respectively, 20, 45, and 36 in inbred K55. If one is interested in obtaining extracts enriched for polypeptides that occur in SF2 and SF3, this could be accomplished easily by extraction of meal with 90% 2-PrOH to remove most of the α-zein.

Conclusions. The extraction of maize prolamin, zein, is faster and more efficient with alcohol containing a reducing agent than one without. Zein so extracted can be separated into three fractions each having a unique polypeptide composition by following one of the two differential solubility fractionation protocols described above. The author believes that this approach provides a better and more rational basis for further studies of zeins. It is also proposed that all alcohol-soluble maize proteins should be referred to as zein whether they require a reducing agent or not for extractability and solubility. The fractions referred to as zein-2, glutelin-1, zein-like, alcohol-soluble glutelin, etc. contain the same polypeptides as does ordinary zein but also the 27 kD proline-rich zein (SF3). These terms have certainly been useful to draw attention to the fact that a part of zein occurs as disulfide-linked oligomers and polymers and could not be extracted without reduction of disulfide bonds. Their continuous use is no longer meaningful and causes much confusion. There would be more clarity about zein nomenclature if everyone followed the usage and criteria originally proposed by Osborne.

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