Extraction and Solubility Characteristics of Zein Proteins from Dry-Milled Corn

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Zein isolation by aqueous ethanol extraction from dry-milled corn produces a mixture of zeins, covalently linked polymers (dimers, tetramers, etc.) and higher-molecular-weight aggregates, some of which were not soluble in aqueous alcohol. The insoluble particles were identified as protein aggregates which form when the extraction solution is heated, particularly under alkaline conditions. The insoluble protein aggregates were not present in zein isolated by the same method from corn gluten meal. Zeins extracted from corn gluten meal and dry-milled corn were fractionated (by differential solubility) to identify differences in their polypeptide compositions. Using polyacrylamide gel electrophoresis, β- and γ-zeins were detected in dry-milled corn, but only trace amounts of β-zein were found in corn gluten meal. Treatment of dry-milled corn with 0.55% lactic acid and 0.2% sulfur dioxide at 50 °C for 6 h before ethanol extraction resulted in a 50% increase in zein isolate yield with high solubility (98%). This pre-extraction treatment cleaved disulfide linkages of the β- and γ-zeins and significantly reduced insoluble aggregates in zein isolates.

Keywords: *Proteins; zeins; polymers; aggregates; disulfide linkages; dry-milled corn; corn gluten meal; pretreatment*

INTRODUCTION

Zein, a prolamine rich protein, is found in protein bodies in the endosperm of the corn kernel. It is a hydrophobic protein widely used for films and coatings. Zein is extracted from corn gluten meal at 60 °C using aqueous alcohol. Less than half of the protein in the meal, however, is isolated (*1*), which increases its cost and thus inhibits new markets. A serial process was studied for producing higher concentrations of zein extract from dry-milled yellow dent corn (*2*). Chromatographic profiles of the alcohol-soluble proteins in zein from ground corn include β- and γ-zeins not present in commercial zein (*3, 4*). These zeins can comprise up to 15% of the total zein in the corn kernel; the remainder are the more hydrophobic α-zein. Commercial steeping of whole corn in sulfur dioxide, though intended to permit separation of germ and fiber from the endosperm, also facilitates starch and protein separation. This process cleaves disulfide bonds linking the alcohol-soluble proteins, thereby disrupting disulfide intermolecularly aggregated native zeins in the corn kernel (*4*−*6*). The objective of this work was to study the influence of β- and γ-zeins on the composition and solution properties of zein extracted from dry-milled corn.

MATERIALS AND METHODS

Materials. Corn, yellow dent, was milled to a particle size of 20 mesh with a counter-rotating ribbed disk mill at Davis Feeds, Perkasie, PA. Corn gluten meal was obtained from a commercial wet milling plant.

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Zein Extraction. Corn zein isolates were prepared by batch extraction with 70% ethanol from dry-milled corn at 60 °C for 2 h according to the method of Dickey et al. (*7*). Zein was also extracted from corn gluten meal with 86% 2-propanol (*1*) or 80% ethanol (*8*).

Chemical Analysis. The protein content ($N \times 6.5$) for zein isolates was determined by the micro-Kjeldahl method (*9, 10*). The starch content was determined according to a previously published procedure (*11*) by measuring the amount of glucose present in trifluoroacetic acid (TFA)-hydrolyzed samples. The total lipid content was determined by packing a glass-wool plugged pipet with approximately 100−300 mg of sample previously dried at 110 °C overnight. The microcolumn was eluted with 5 mL of hexane followed by 5 mL of chloroform. The eluates were collected in a tared vial and subsequently evaporated to a constant weight with a stream of nitrogen gas, and the weights of hexane and chloroform extracts were determined. The relative standard deviation of the method was 2.9% ($n = 5$). Lipid components in crude-filtered hexane extracts from zein isolates were separated and quantified by a previously published method (*12*). The separation was carried out on a 5 mm (3 × 100 mm) LiChrosorb DIOL column using a ternary gradient system consisting of hexane/2-propanol/acetic acid and a constant flow rate of 0.5 mL/min. Eluting peaks were detected with an Alltech-Varex Mark III evaporative light scattering detector (ELSD) at 40 °C with nitrogen as the nebulizing gas at a flow rate of 1.6 L (STP)/min.

Fractionation of Zein. Zein proteins in corn and corn gluten meal were fractionated on the basis of solubility using a modification of the method described by Esen (*13*). Two grams of dry-milled corn (DMC) or corn gluten meal (CGM) was extracted 4 times at room temperature with 20 mL of 60% 2-propanol (2-PrOH) plus 1% 2-mercaptoethanol (2-ME). Three volumes of 100% 2-PrOH were added to the extract and the resulting solution was left standing overnight at 4 °C. The solution was then centrifuged at 12000×*g*, at 4 °C, for 10 min. The pellet containing the β- and γ-zeins was analyzed by gel electrophoresis. Two volumes of water were added to the supernatant, followed by 0.01 volumes of 3 mM sodium acetate

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(pH 6.0). The solution was stored overnight at 4 °C then centrifuged at 5000×*g*, 4 °C, for 10 min. The supernatant was discarded and the pellet containing the α-zein was analyzed by gel electrophoresis.

Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of corn proteins was carried out on a Phast System Pharmacia (Piscataway, NJ) with a phast gel of 20% acrylamide. Dried samples were solubilized in 200 μL of protein solvent system (0.44 M Tris, 1 mM EDTA, 10% SDS, pH 8.0) plus 40 μL of 2-ME, and the mixtures were heated at 100 °C for 10 min. Deionized water replaced 2-ME in the protocol for samples not reduced. Gels were stained with 0.2% (w/v) Coomassie R350 dye. Molecular weight standards (Bio-Rad, Richmond, CA) and their corresponding molecular weights were as follows: phosphorylase b, 97,000; bovine serum albumin (BSA), 66,200; ovalbumin, 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

Solubility in Aqueous Alcohol. Solubility of the zein samples was determined in 90% ethanol, which is the solvent used to prepare zein films. One gram of zein was dissolved in 30 mL of 90% ethanol with gradual heating to 70 °C, over 10 min. Insoluble particles were removed by filtration. The filtrate was dried to constant weight in a vacuum oven at 50 °C. Solubility was determined by measuring the weight of dry matter remaining and reported on initial dry weight basis.

Film Formation. One gram of the zein isolate was dissolved in 30 mL of 90% ethanol. The mixture was heated with stirring at 70 °C for 10 min, then cast in polystyrene Petri dishes and dried in a vacuum oven adjusted to 33.75 kPa at 50 °C. The dried films were stored in a desiccator at 52% relative humidity for 24 h before evaluation.

Tensile Property Measurements. Tensile properties were determined using an Instron model 1122 tensile tester with a 2000 g load cell (*14*). Samples were conditioned overnight in a desiccator held at 52% RH until just prior to placement in the tensile tester jaws.Tensile strength measurement data were collected and analyzed using the DOS-based Series IX, Version 6, Instron software.

Pretreatment of Dry-Milled Corn. A mixture of 40 g of dry-milled corn in 200 mL of 0.5% sulfuric acid was stirred at 50 °C for 6 h. The insoluble corn was separated from the dilute acid by filtration, then mixed and rinsed with 200 mL of water. The corn was again collected by filtration, and the filtrate was discarded. The washed, acid-treated corn was then extracted with 70% ethanol, as described earlier, after correcting for its moisture content. Dry-milled corn was similarly treated with 0.5% sodium sulfite, 0.5% sodium bisulfite, or 0.55% lactic acid plus 0.2% sulfur dioxide (*15*).

RESULTS AND DISCUSSION

Zein isolated by aqueous ethanol extraction from dry-milled corn contained a mixture of zeins, covalently linked polymers, and higher-molecular-weight aggregates. Others have shown that the major storage protein, α-zeins, is soluble in 95% ethanol; the minor proteins β-, γ-, and δ-zeins are soluble in 60% ethanol but not in 95% ethanol (*16*). Figure 1 shows an electrophoretic profile of zein extracted with different compositions of aqueous ethanol. Zein isolated by extraction with 95% ethanol (lane 2) is composed of primarily α-zeins (22 and 24 kDa), and their dimer and tetramer. Zein extracted with 90% and 80% ethanol contained more of the higher-molecular-weight polypeptides, and a small amount of β-zein (18 kDa) was present in the isolate extracted with 80% ethanol. Zein extracted with 70 and 60% ethanol contained more aggregates sufficiently large that they did not enter the running gel, and other larger aggregates that did not migrate in the stacking gel. Zein polymers and aggregates are not soluble in 50% ethanol (lane 7), and the amount of

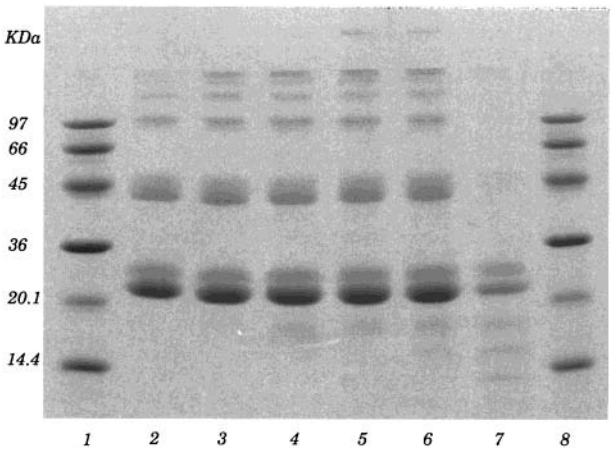

Figure 1. SDS−polyacrylamide gel electrophoresis (not reduced) of zeins extracted from dry-milled corn with aqueous ethanol. Lanes 1 and 8, molecular weight standards; lane 2, 95% ethanol; lane 3, 90% ethanol; lane 4, 80% ethanol; lane 5, 70% ethanol; lane 6, 60% ethanol; lane 7, 50% ethanol.

Table 1. Solubility of Zein Extracted from Dry-Milled Corn[a,b]

extraction conditions	(%)[c]
70% ethanol, 60 °C, 2 h	76
70% ethanol, 23 °C, 2 h	95
70% ethanol, 23 °C, 2 h, 0.25% NaOH	96
70% ethanol, 60 °C, 2 h, 0.25% NaOH	64

[a] In 90% ethanol. [b] Solubility measurements were done in duplicate. [c] Percentage of zein in solution.

α-zein in this isolate was significantly reduced. In addition to the β-zein two other low-molecular-weight polypeptides were present; one band was attributed to δ-zein (10 kDa). For this study extractions were carried out using 70% ethanol to maximize the amount of zein isolated and to evaluate the product of a practical extraction in which it would be important to minimize the amount of ethanol used and the cost of recovering it for reuse.

Extraction of dry-milled corn with 70% ethanol at 60 °C for 2 h produced a solution of about 2% of zein. Typically this isolate was composed of about 80−85% protein; 15−20% lipid; and <0.25 starch. The principal lipid components were free fatty acids because most of the triacylglycerides were removed with the insolubles during centrifugation of the extract. Typically, the lipid composition of zein isolates was 80% fatty acids, 10% triacyglycerides, 5% phytosterols, and 0.5% phytosterol esters. The isolate was a yellow powder with the characteristic corn aroma. When cast from a 90% ethanol solution, it formed a clear flexible film after insoluble particles were removed by filtration. Flexibility of the films was attributed to the endogenous linoleic, oleic, and palmitic fatty acids (*17*).

The zein isolate was not completely soluble in aqueous alcohol. Only 76% of the isolate extracted at 60 °C was soluble in 90% ethanol (Table 1). It appeared that the insoluble particles were residual corn hull and fiber; however, when extractions were carried out at 23 °C the solubility of the isolate increased to 95%. Addition of alkali to the extraction solution did not significantly affect the solubility of the isolate except at 60 °C (Table 1). It appears that the insoluble particles are protein aggregates that form when heated, particularly under alkaline conditions. These protein aggregates are associated primarily through disulfide bonds because

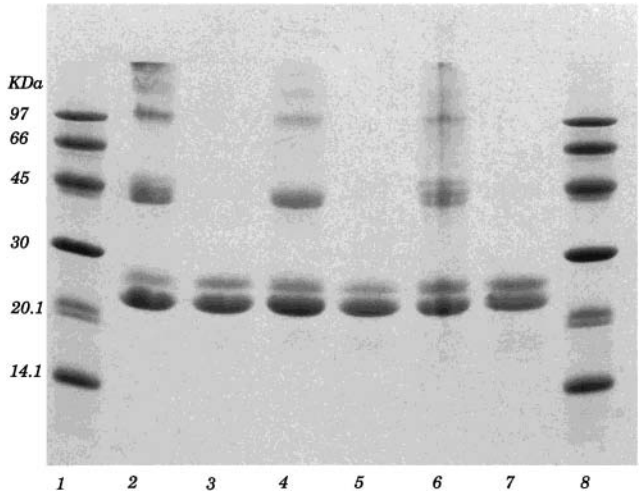

Figure 2. SDS−polyacrylamide gel electrophoresis of zeins extracted with 70% ethanol from dry-milled corn. Lanes 1 and 8, molecular weight standards; lanes 2 and 3, extracted at 23 °C; lanes 4 and 5, extracted at 60 °C; lanes 6 and 7, extracted at 60 °C plus 0.25% NaOH. Samples were reduced in lanes 3, 5, and 7 and not reduced in lanes 2, 4, and 6.

Table 2. Solubility of Zein from Corn Gluten Meal[a,b]

extraction conditions	(%)[e]
88% 2-propanol 60 °C, 2 h, 0.25% NaOH[c]	100
80% ethanol, 60 °C, 2h[d]	92

[a] In 90% ethanol. [b] Solubility measurements were done in duplicate. [c] Reiners et al. (*1*). [d] Cooke et al. (*8*). [e] Percentage of zein in solution.

samples reduced with 2-ME contained only α-zein monomer (Figure 2; lanes 3, 5, and 7). Although various attempts were made to completely dissolve the samples in the Tris−SDS buffer, none was successful because extractions carried out at 23 °C (lane 2) indicated more protein aggregation than extractions carried out at 60 °C (lanes 4 and 6). Also see Table 1.

It has been suggested that some properties of zeins extracted directly from corn with alcoholic solutions cannot be observed in commercial zeins because of disruption of intermolecular disulfide bonds (*18*). To determine whether insoluble aggregates form in zein extracted from corn gluten, we carried out the extraction using two different processes. The most commonly used method (*1*) was extraction with 88% 2-propanol containing 0.25% NaOH (Table 2). After extracting corn gluten with this solution at 60 °C for 2 h, the solution was decanted and dried. Zein isolated in this way was completely soluble in 90% ethanol. Following the method of Cook et al. (*8*), zein was extracted with 80% ethanol. After 2 h at 60 °C insoluble particles were removed by filtration. Zein was precipitated from the filtrate by reducing the alcohol content to 40%, and the solution was held overnight at 4 °C. The zein was recovered by centrifuging at 10000×*g*, 4 °C, for 15 min. Using this method, 92% of the dried precipitate was soluble in 90% ethanol. Using the latter conditions, unfolding of the molecules during precipitation may have resulted in formation of noncovalent associations and decreased solubility.

To identify differences in the polypeptide composition of corn gluten meal and ground corn, a modified fractionation scheme originally developed by Esen (*13*) was used to separate the zeins on the basis of solubility (Figure 3). CGM or DMC was extracted four times with

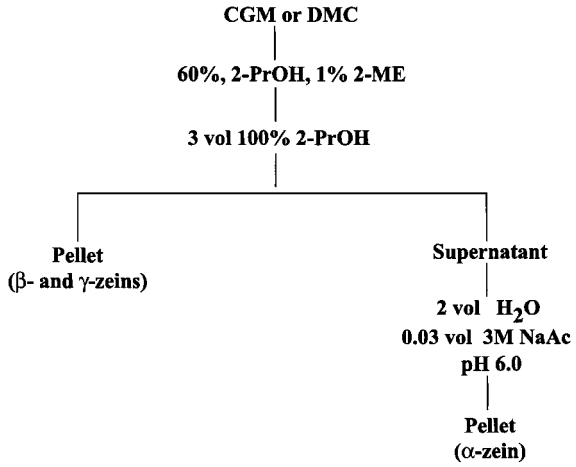

Figure 3. Fractionation of zein proteins in corn gluten meal (CGM) and dry-milled corn (DMC).

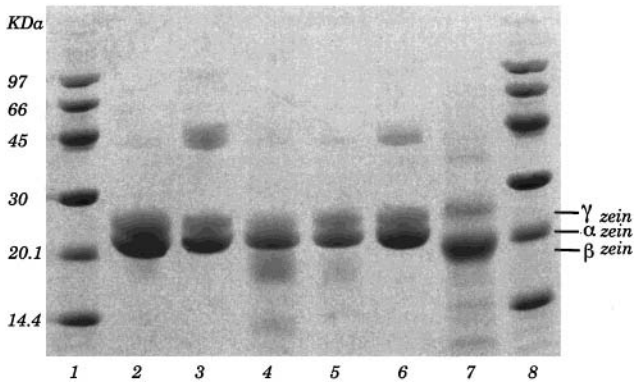

Figure 4. SDS−polyacrylamide gel electrophoresis of zein proteins in CGM and DMC. Lanes 1 and 8, molecular weight standards; lanes 2−4, CGM; lanes 5−7, DMC; lanes 3 and 6, soluble fractions; lanes 4 and 7, insoluble fractions.

60% 2-PrOH containing 1% 2-ME for complete extraction of the zein proteins. Addition of 3 volumes of 100% 2-PrOH increased the alcohol concentration to 90% PrOH. At this concentration the α-zeins are soluble but the *β*- and *γ*-zeins are not. SDS−polyacrylamide gel patterns of the alcohol-soluble polypeptides in CGM and DMC appeared to be similar (Figure 4; lanes 2 and 5) and consisted almost exclusively of α-zeins. Other zeins are not present in quantities sufficient to be detected. After the extraction solution was adjusted to 90% PrOH, the fraction containing the soluble zeins for CGM and DMC were similar (lanes 3 and 6) consisting of α-zeins and their dimers. The insoluble fraction, however, was not similar for the two samples. Densitometry scans indicated that the 90% PrOH insoluble fraction of DMC corn consisted primarily of 77% *β*-, 10% *γ*-, and 3% *δ*-zeins (lane 7). The similarly insoluble fraction for CGM consisted of 78% α-, and 12% *β*-zeins, and 5% of an unknown protein band, 14 kDa (lane 4). The absence of *γ*- and significantly reduced amounts of *β*-zeins in CGM agrees with previous findings by Parris et al. (*3*) and Wolf and Lawton (*4*). It appears that these minor proteins, which are rich in disulfide bonds, were cleaved during the sulfur dioxide steeping and were removed from the corn with the steep liquor or made insoluble in the corn.

Treatment of DMC with 0.5% sulfuric acid at 50 °C for 6 h before ethanol extraction was sufficient to cleave disulfide linkages of the *β*- and *γ*-zein. Zein extracted from this pretreated corn was recovered in 1.8% yield

Table 3. Recovery and Solubility of Zein Extracted from Pretreated Dry-Milled Corn[a,b]

pretreatment	zein isolate (%)[c]	solubility (%)[d]	film characteristics
0.5% sulfuric acid	1.8	94	clear/smooth
0.55% lactic acid + 0.2% sulfur dioxide	2.7	98	clear/smooth
0.5% sodium bisulfite	1.3	96	cloudy/rough
0.5% sodium sulfite	2.1	50	cloudy/rough

[a] In 90% ethanol. [b] Experiments were done in duplicate. [c] Percentage of protein recovered from DMC. [d] Percentage of zein in solution.

Table 4. Tensile Properties of Zein Films from Pretreated Dry-Milled Corn[a]

pretreatment	tensile strength	elongation to break	modulus
0.5% sulfuric acid	15.7 (1.8)	3.0 (0.3)	694 (77)
0.55% lactic acid + 0.2% sulfur dioxide	19.5 (3.6)	3.1 (0.3)	550 (66)
0.5% sodium bisulfite	13.5 (3.0)	3.2 (0.3)	568 (80)
0.5% sodium sulfite	9.2 (1.4)	2.4 (0.5)	663 (28)

[a] Values in parentheses indicate standard deviation

and had good solubility (94%) in aqueous ethanol (Table 3). Treatment of DMC with a mixture of 0.55% lactic acid plus 0.2% sulfur dioxide improved the recovery of zein isolate by approximately 50% to 2.7%, and its solubility in aqueous ethanol was 98%. Both pretreatments of DMC resulted in a zein isolate with good film-forming properties (Table 3). Pretreatment with sodium bisulfite or sodium sulfite resulted in an isolate which produced films of poor clarity and smoothness. It appears that pretreatment of DMC with sulfuric acid or lactic acid plus sulfur dioxide cleaves native disulfide linkages in corn as does commercial steeping of corn in sulfur dioxide.

The strength and flexibility of zein film from pretreated DMC (Table 4), was measured by its tensile strength (TS) and elongation to break (ETB). Initial modulus is a measure of resistance to stretching, or stiffness. In general, films prepared from zein isolated from DMC pretreated with 0.55% lactic acid plus 0.2% sulfur dioxide were stronger than, less stiff than, and as flexible as the other pretreated films. Films prepared from zein isolated from DMC pretreated with 0.5% sodium sulfite, however, exhibited the lowest TS and ETB values of the films tested.

Results from this study should be useful to producers of zein lipid mixtures.

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