

Disulfide Structures of Zein Proteins from Corn Endosperm

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ABSTRACT

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Zein, the prolamine fraction of corn protein, consists of a series of disulfide-linked oligomers varying in molecular weight and intramolecular disulfide-bonded polypeptides, as demonstrated by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Whole native zein from normal corn was fractionated with 95% ethanol to obtain a soluble α -fraction and an insoluble β -fraction. The unreduced α -fraction consisted of a series of oligomers; SDS-PAGE revealed a prominent band at a molecular weight of 24,000. The native α -component was heterogeneous by both PAGE at pH 3.5 and by isoelectric focusing between pH 6 and 9. The β -component did not migrate into the gel during

SDS-PAGE and showed only a streak following PAGE at pH 3.5 because it was too high in molecular weight. The α - and β -fractions after reduction of disulfides exhibited both 22,000 and 24,000-mol wt subunits upon SDS-PAGE. Native zein from high-lysine corn (*opaque-2*) contained no 22,000-mol wt monomers. However, reduced *opaque-2* zein contained 22,000-mol wt subunits but had a smaller proportion of 24,000-mol wt subunits than did normal zein. Fundamental compositional and functional differences in the 22,000 and 24,000-mol wt categories of zein polypeptide chains may result in differences in properties of normal and high-lysine zeins.

Turner et al (1965) resolved zein by starch gel electrophoresis into several well-defined components, but some high molecular weight protein remained at the origin. The α -zein (the fraction soluble in 95% ethanol) migrated as distinct components during starch gel electrophoresis; however, the 95% ethanol-insoluble β -zein remained at the origin, due to its high molecular weight. After reduction of disulfide bonds, all zein subunits can migrate into starch gel upon electrophoresis, showing that disulfide bond cleavage reduces zein size (Turner et al 1965). Zein also was fractionated according to molecular size by gel filtration chromatography on Sephadex G-200 (Ganchev et al 1976). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the fractions they obtained indicated that the higher molecular weight zeins are polymers of the lowest molecular weight fractions. Landry (1979) found that native zein consists primarily of a protein having mobility near 22,000 mol wt and three or four proteins with an average molecular weight of 45,000. Reduction of disulfides in these proteins yielded subunits with molecular weights of 22,000 and 24,000.

To establish the basis for variations in properties of zein solubility fractions, I compared native and reduced α -zeins and β -zeins from hybrid dent corn. Yields, amino acid compositions, PAGE, molecular weight by SDS-PAGE, isoelectric focusing (IEF) patterns, and gel filtration behavior were determined on the various zein fractions and their disulfide-cleaved forms. Electrophoretic patterns of zeins from near-isogenic lines of normal, *opaque-2* (o_2), and *floury-2* (fl_2) corns were also compared by these methods to determine whether any differences occurred between their native and alkylated-reduced forms. These studies confirm the concept that intramolecular and intermolecular disulfide bonds are important in modifying properties of zein polypeptides and indicate that different polypeptides show

different tendencies to participate in intramolecular and intermolecular disulfide bonds. These variations may contribute to the different properties of normal and high-lysine zeins.

MATERIALS AND METHODS

Preparation and Solubilities of Zeins

The preparation of defatted normal corn endosperm meal used for zein fractionation studies was described by Paulis and Wall (1971). The zein proteins from high-lysine corns were isolated from defatted endosperm meals of the near-isogenic lines P-A-G 50001 o_2 and P-A-G 50101 fl_2 derived from a normal hybrid corn, P-A-G SX52 (Paulis et al 1975). Zein was extracted from the endosperm meals with 70% ethanol containing 0.5% sodium acetate by previously described methods (Paulis and Wall 1971, Paulis et al 1975). To obtain α -zein, approximately 1 g of whole zein prepared from normal endosperm meal was extracted with 100 ml of 95% ethanol according to the procedure of Turner et al (1965). The insoluble material is β -zein.

Solubilities of native and reduced α -zeins and β -zeins were determined by sonicating dispersions in tubes containing 0.9 mg of N in 10 ml of 8M urea with and without 1% β -mercaptoethanol (ME) with a Cole-Parmer ultrasonic cleaner for 3 min (Huebner and Rothfus 1971). The dispersions were centrifuged at 2,000 \times g for 10 min, and the absorbances of the supernatants were measured at 280 nm on a Beckman D.U. spectrophotometer. Solubilities were determined as A_{280} relative to A_{280} of an equal amount of α -zein, which appeared totally soluble in the solutions. No corrections were made for effect of light scattering on the absorbance at 280 nm.

Analytical Methods

Samples were analyzed for nitrogen by a semimicro Kjeldahl method and for amino acids as described elsewhere (Paulis and Wall 1977). The amino acid analyses are accurate to $\pm 5\%$. Cystine and methionine values were determined only on HCl hydrolysates. The values for methionine represent a summation of methionine, methionine sulfoxide, and methionine sulfone as determined by the analyzer.

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The proteins were reduced with 1% ME in 8M urea overnight at room temperature and alkylated with acrylonitrile (Paulis and Wall 1977). The cyanoethylated and native proteins were dissolved in 8M urea and separated by PAGE in aluminum lactate buffer, pH 3.5, on 5% gels containing 8M urea as described previously (Paulis and Wall 1977).

Proteins (2.5 mg) were reduced and equilibrated for SDS-PAGE by heating the samples at 95°C for 5 min in 0.1 ml of borate buffer (pH 8.9) containing 5.1% SDS and 1% ME. The unreduced proteins were treated similarly in SDS containing borate buffer without ME before SDS-PAGE. Molecular weights of reduced and unreduced zeins were estimated by comparing the SDS-PAGE mobilities in 10% gel in 0.1% SDS-borate buffers (pH 8.9) to mobilities of known reduced proteins (Paulis and Wall 1977). Molecular weights for standard proteins are cytochrome C, 11,700; ribonuclease, 13,700; chymotrypsinogen, 25,700; ovalbumin, 43,000; and bovine serum albumin, 68,000.

IEF was performed according to a method described by Gianazza et al (1976) on thin polyacrylamide gels in 8M urea with ampholytes giving a pH range of 6-9 at 13 W constant power.

Gel Filtration

To prepare pyridylethyl (PE) zein for gel filtration, 2 g of zein was first reduced for 16 hr in 100 ml of 6 M guanidine/HCl/0.133M tris buffer (pH 8) containing 1% ME and 0.1% ethylenediaminetetraacetic acid. The sulfhydryl groups of the protein were alkylated with 14 mmoles of 4-vinyl-pyridine for 2 hr. The solution then was adjusted to pH 3 with acetic acid, exhaustively dialyzed against water, and lyophilized to dryness.

Fractionation of whole, α -, and PE-zeins was achieved on a 2.5 x 90-cm column packed with Sephadex G-200, which eluted upward at 12 ml/hr with 8M urea. All samples added to the columns were dissolved in 5 ml of 8M urea to give an approximately 1% solution (6.6-7.3 mg of N) and introduced at the same flow rate used for subsequent elution. Five-milliliter fractions were collected and

monitored by reading their absorbance at 280 nm on a Beckman D.U. spectrophotometer. The hold-back volume of the column for proteins that exceeded the maximum molecular weight for entrance into gel pores (200,000 mol wt) was 130 ml.

RESULTS AND DISCUSSION

Solubility and Composition

Yields of normal corn zein and its α - and β -fractions and their solubilities in 8M urea with and without ME are given in Table I. Zein's α -component (35% of whole zein) was completely soluble in 8M urea; whole zein and β -zein were only 58 and 20% soluble, respectively. Apparently, extraction, dialysis, and lyophilization of β -zein decreases its solubility in 8M urea as compared to the solubility of β -zein in total zein extracts, possibly due to disulfide interchange resulting in more intermolecular disulfide bonds. Inclusion of 1% ME in 8M urea increased the solubility of whole and β -zein to 86 and 80%, respectively, due to disruption of their disulfide bonds. The amount of β -zein solubilized after disulfide bond reduction is almost the same as that of total zein.

Significant differences in amino acid composition are observed between α - and β -zeins (Table II). β -Zein contains more histidine, arginine, proline, and methionine than does α -zein. These amino acids are among those occurring in larger quantities in alcohol-soluble reduced glutelin (ASG) than in zein (Paulis and Wall 1977), suggesting that the different compositions of α - and β -zeins might be related to some ASG polypeptides in β -zein.

Gel Electrophoresis of Fractionated Zeins

SDS-PAGE patterns of zein and its α - and β -fractions are shown in Fig. 1. The whole and α -zein consisted mainly of 45,000 and 68,000-mol wt bands, with a prominent band having a molecular weight of 24,000. Native β -zein did not migrate into the gel during electrophoresis. After reduction, α - and β -zeins exhibited both 22,000 and 24,000-mol wt subunits. Data presented by Ganchev et al (1976) suggest that the 22,000-mol wt subunits may exist

TABLE I
Relative Yields and Solubilities of Protein Fractions from Zein

Fraction	Total Protein Extracted (%) ^a	Percent Soluble ^b in	
		8M Urea	8M Urea-1% ME ^c
Whole zein	40.7	58	86
α -Zein	14.3	100	100
β -Zein	26.4	20	80

^a Percent of endosperm meal protein.

^b Determined by A_{280} relative to A_{280} of an equal amount of α -zein, which was considered 100% soluble.

^c β -Mercaptoethanol.

TABLE II
Amino Acid Composition^a of Zein Preparations

Amino Acid	Total Zein	α -Zein	β -Zein
Lysine	0.1	0	0.2
Histidine	2.6	1.8	3.3
Ammonia	4.4	5.1	3.9
Arginine	3.1	2.6	3.6
Aspartic acid	4.2	4.4	3.9
Threonine	2.6	2.2	2.7
Serine	4.5	4.4	4.6
Glutamic acid	24.2	23.1	24.8
Proline	8.9	7.1	10.1
Glycine	1.3	0.9	1.8
Alanine	8.4	7.9	8.0
Valine	3.4	3.1	3.5
Methionine	1.3	0.5	2.4
Isoleucine	3.5	3.2	3.4
Leucine	17.7	17.0	17.0
Tyrosine	4.3	3.3	4.9
Phenylalanine	6.0	5.6	5.7

^a In g/100 g of protein.

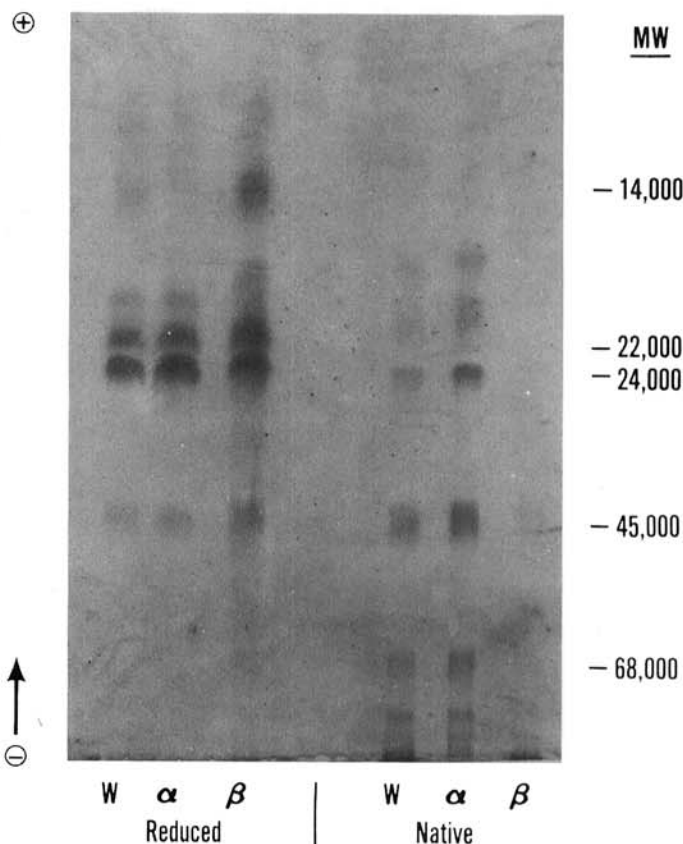


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of native and reduced whole zein (W), α -zein (α), and β -zein (β).

primarily in intermolecular disulfide-linked chains in native zein as oligomers represented by the 45,000, 68,000, and higher molecular weight bands. Alternatively, both 22,000 and 24,000-mol wt subunits may migrate at the same rate in their native form when folded, so that their complexes with SDS are similar. A small amount of 45,000-mol wt protein appeared in the reduced samples; either reduction is incomplete or a minor 45,000-mol wt polypeptide exists. A protein band with a molecular weight of approximately 14,000 occurred in reduced β -zein. This band may be low molecular weight ASG polypeptides (Paulis and Wall 1977),

which may account for the β -fraction's slightly different amino acid composition than those of whole and α -zeins. Gianazza et al (1977) observed that the 14,000-mol wt protein differed in amino acids, containing high levels of methionine.

Native whole and α -zeins exhibit about nine components by PAGE (Fig. 2). Again, most of the β -fraction did not enter the gel but streaked from the origin, probably due to high molecular weight and poor solubility. After reduction and alkylation with acrylonitrile, whole and β -zeins migrated completely into the gel and exhibited a fast band in region 1 that was missing in the α -fraction. This band may be due to the presence of the subunits also found in ASG (Paulis and Wall 1977). The two fast-moving bands in region 2 in all the reduced zeins seem to appear at a slightly lower mobility than that of the corresponding ones in the same region in their native forms. This difference in mobility may be due to unfolding of the protein after reduction. Region 3 contains bands with similar mobilities in both native and reduced zeins. Reduction and alkylation (R-A) of disulfide bonds eliminated all of the streaking in the gel caused by high molecular weight protein in native zeins. The distinct bands of native whole and α -zein that enter into the gel probably are derived from free 24,000-mol wt and 17-20,000-mol wt proteins (Fig. 1). The streaked PAGE (Fig. 2) portion of native zeins may be due to oligomers containing 22,000 and 24,000-mol wt subunits.

IEF resolved approximately 15 R-A zeins (Fig. 3) compared to about nine by PAGE. Significant differences occurred between IEF patterns of α - and β -zeins, indicating some qualitative and quantitative differences in subunits of the two proteins. Some bands in native whole and α -zeins are seen at isoelectric points corresponding to those of the R-A forms. However, some bands in native zein are not prominent in the R-A zein, and bands with different isoelectric points appear after reduction. Possibly this difference is due to variations in IEF points of some dimers. Again,

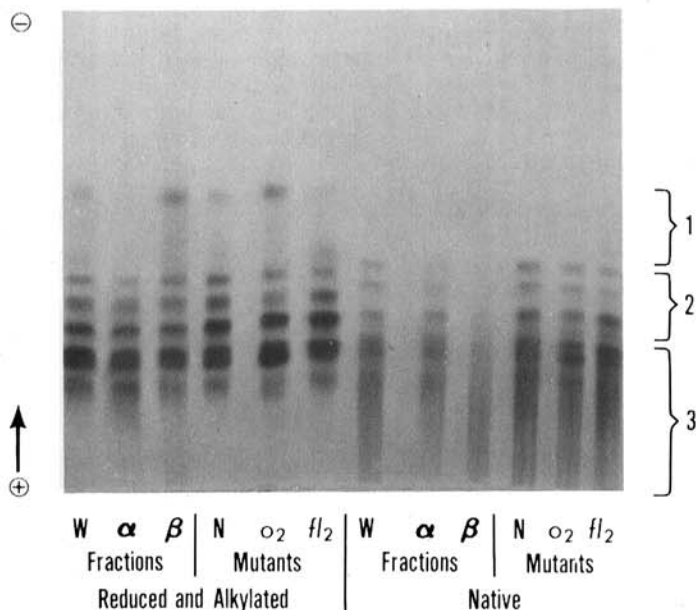


Fig. 2. Polyacrylamide gel electrophoresis patterns of native and reduced and alkylated (cyanoethylated) patterns of whole zein (W), α -zein (α), β -zein (β), and zeins from normal SX52 corn endosperm (N) and its *opaque-2* (o_2) and *floury-2* (fl_2) lines.

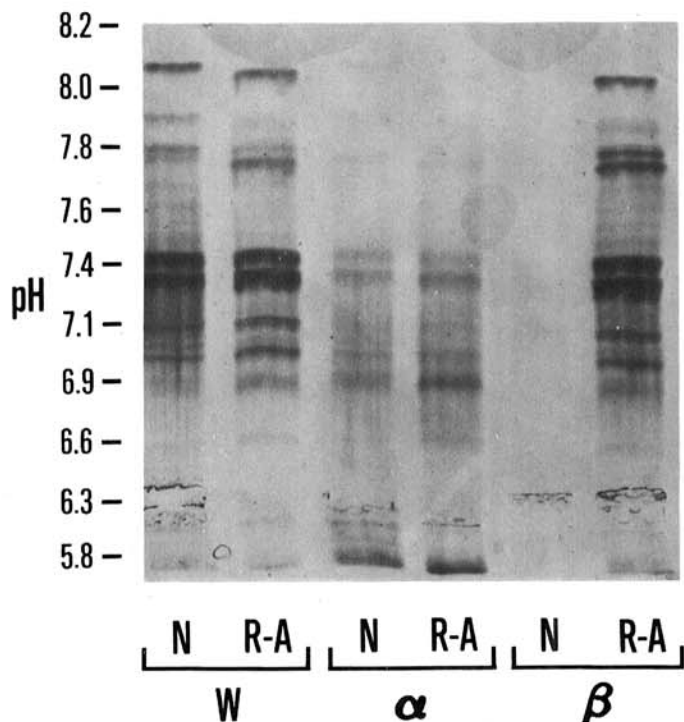


Fig. 3. Polyacrylamide gel isoelectric focusing patterns of native (N) and reduced-alkylated cyanoethylated (R-A) whole zein (W), α -zein (α), and β -zein (β). Measured pH at gel shown on left.

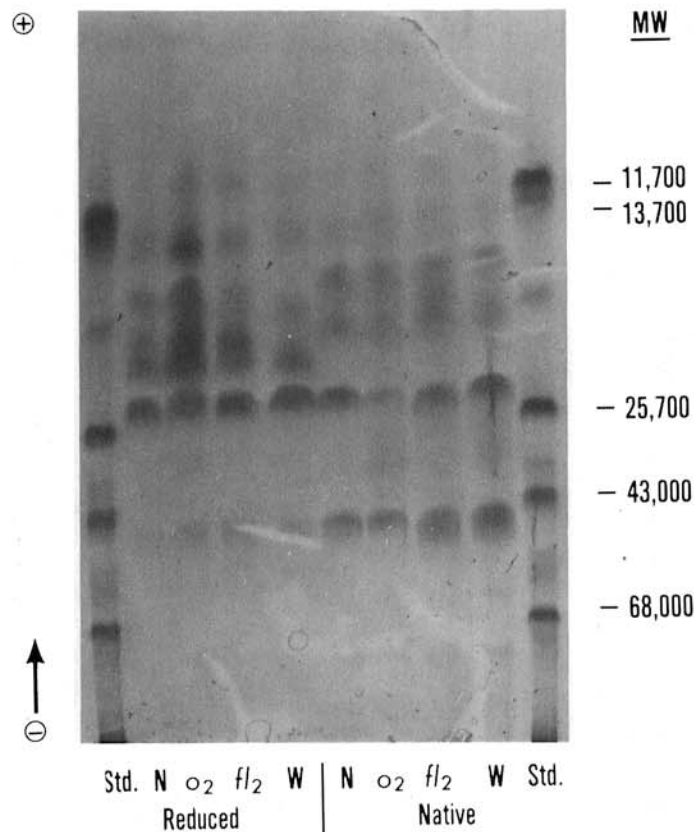


Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of molecular weight calibration mixture (Std), whole zein (W), and zeins from a normal corn endosperm (N) and its *opaque-2* (o_2) and *floury-2* (fl_2) near-isogenic lines. Standards are cytochrome C, 11,700; ribonuclease, 13,700; chymotrypsinogen, 25,700; ovalbumin, 43,000; and bovine serum albumin, 68,000.

native β -zein does not migrate into the gel until it is solubilized and its molecular weight reduced by breaking of its disulfide crosslinks.

Gel Electrophoretic Comparison of Mutant Endosperm Proteins

SDS-PAGE and PAGE confirm that whole zeins from the endosperms of the near-isogenic normal, o_2 , and fl_2 corns are qualitatively similar to the zein patterns from normal endosperm (Figs. 2 and 4), but some quantitative differences may occur. Native zeins from all mutant endosperms also lack protein migrating as 22,000-mol wt and contain 24,000-mol wt monomers (Fig. 4). The 24,000-mol wt subunits are present in smaller amounts in o_2 native zeins than in native zeins from the normal and fl_2 counterpart endosperms. Upon reduction, the 45,000-mol wt band is almost completely eliminated and a 22,000-mol wt band appears. Again, reduced o_2 zeins have smaller amounts of the 24,000-mol wt subunits than do the zeins from other genotypes. A larger amount of protein was applied to the reduced o_2 pattern shown in Fig. 4, which exaggerated the apparent relative amount of the 24,000-mol wt subunit and of the 14,000-mol wt subunit. Because o_2 contains more glutelin protein, some additional 14,000-mol wt protein may come from ASG. This decrease in 24,000-mol wt subunits in reduced o_2 zein compared to the quantity in the normal unreduced zeins (Fig. 4) has been observed by others (Lee et al 1976, Misra and Mertz 1976). These fundamental quantitative differences in the two major molecular weight categories of zein polypeptide chains may result in differences in properties of normal and o_2 zeins.

Overall, the native zeins of the near-isogenic endosperms that migrate into the gel give the same patterns by PAGE as their reduced forms do (Fig. 2).

Therefore the oligomers contributing to the unmobile and streaked region in the pattern of the unreduced native samples are composed of the same polypeptides as the monomers that give discreet bands in the native protein patterns, although they are possibly in a different quantitative relationship. Between patterns of zeins from different near-isogenic endosperm genotypes, some quantitative differences occur in regions 1 and 2 with native and reduced proteins.

Gel Filtration

The elution profile of zein from the Sephadex G-200 column (Fig. 5a) is consistent with the electrophoretic pattern in SDS-PAGE (Fig. 1) and with the pattern in PAGE at pH 3.5. Peak D is

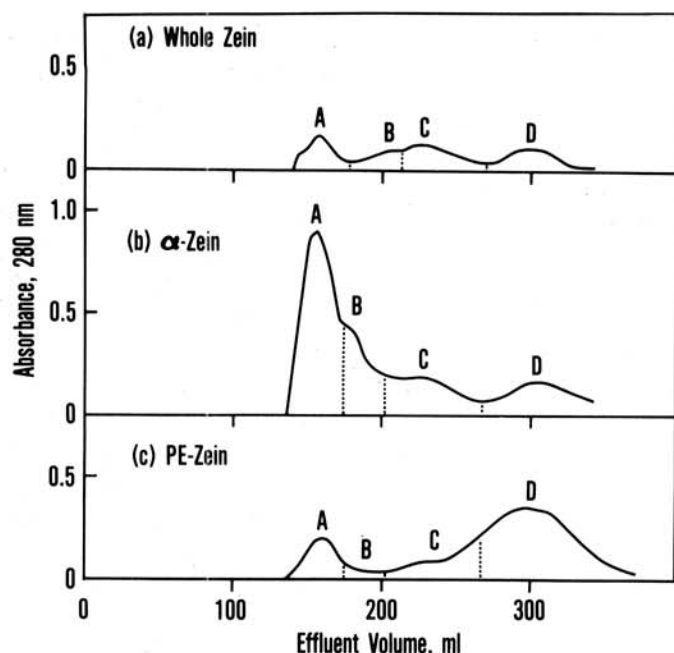


Fig. 5. Fractionation of: a, whole zein; b, α -zein; c, reduced-pyridylethylated zein on Sephadex G-200. Broken-lined areas represent fractions combined for analysis.

composed of 22,000 and 24,000-mol wt subunits; peak C of 45,000, peak B of 68,000, and peak A of high molecular weight constituents. The origin of the large amount of high molecular weight oligomer in α -zein (Fig. 5b) is not known because the whole zein contains a smaller proportion of high molecular weight proteins. Probably the additional extraction and dialysis of zein resulted in strong hydrophobic association or disulfide interchange to yield more high molecular weight oligomer. The α -zein separation in Fig. 5b resolves the 22,000 and 24,000-mol wt subunits in peak C and also separates dimer (peak C) and the higher molecular weight oligomers (peak A), which predominate. SDS-PAGE establishes that after reduction, each of these fractions is converted to lower molecular weight components, predominately of 22,000 and 24,000 molecular weight (Fig. 6). The PE-zein consisted mainly of 22,000 and 24,000-mol wt subunits with a small amount of residual 45,000 and higher molecular weight material, as shown by its fractionation on Sephadex G-200 (Fig. 5c).

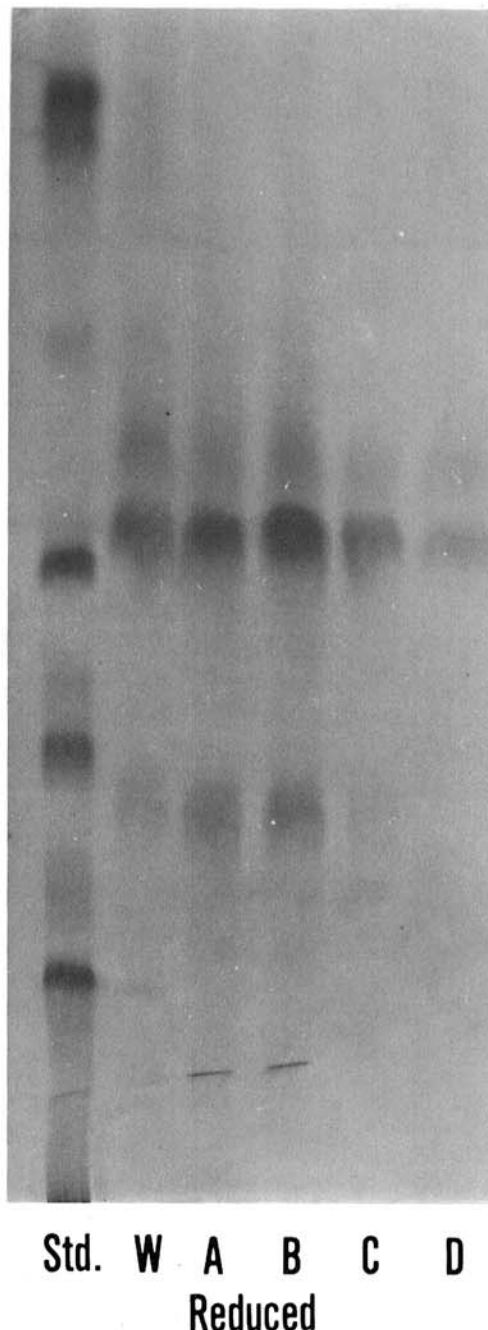


Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of whole zein (W) and α -zein fractions from Sephadex G-200 chromatography: A-D, peaks A-D, respectively, in Fig. 5b.

CONCLUSIONS

SDS-PAGE and gel filtration studies in this investigation substantiate earlier observations of Turner et al (1965), Landry (1965, 1979), and Ganchev et al (1976) that native zein occurs as single polypeptide chains and disulfide-linked oligomers. Whether the zein occurs in this form in situ in tissues or this complex results from disulfide exchange during alcohol extraction was not established. α -Zein consists of monomers and a series of oligomers of increasing molecular weight, whereas β -zein consists of higher molecular weight oligomers. The amount of high molecular weight zein extracted from meal in the present study may be higher than that observed by Landry (1979) because we incorporated sodium acetate in the 70% ethanol extracting media. Sodium acetate was used because it was previously shown to extract more proteins (Paulis et al 1969). Landry extracted zein from corn with 92% ethanol, which extracted little high molecular weight protein and thereby yielded a more homogeneous product. The monomers in our native zein have molecular weight around 24,000. This value differs from that of Landry (1979), who estimates that the monomer of native zein has a molecular weight of 22,000. Upon reduction, the oligomers yield both 22,000 and 24,000-mol wt subunits. The same relationship holds for o_2 and f_1 corn zeins, but o_2 zein contains less monomer and less 24,000-mol wt protein subunits. The 24,000-mol wt and 22,000-mol wt subunits are very heterogeneous, in terms of amino acid sequences, as demonstrated by separation of whole reduced zein into 9 and 15 components by PAGE and IEF, respectively. Probably, the 22,000-mol wt subunits have a greater tendency to form oligomers, and the variation in concentration of these proteins in o_2 and normal corns may result in some differences in the physical properties of their zeins.

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