Polymorphism of Native Zein as Detected by Gel Filtration and Electrophoresis in the Presence or Absence of Sodium Dodecyl Sulfate[1]

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ABSTRACT

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Unreduced zein, isolated by extracting maize grains with aqueous isopropyl alcohol and subjected to sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis, is composed of several bands with mean molecular weight (mol wt) of 45,000 and two bands of 24,000 and 22,000 mol wt. When subjected to gel filtration on Ultrogel Ac44 in 1.5% SDS, zein was resolved into two main fractions. The first fraction consisted mostly of proteins with mol wt near 45,000. The second fraction contained a small amount of protein in 45,000 mol wt that eluted early, but consisted mostly of 24,000 mol wt protein that also eluted early, and 22,000 mol wt protein. When reduced, the two gel fractions contained only 24,000 and 22,000 mol wt components, but the percentage of 24,000 mol wt component was higher in the first than in the second fraction. The 24,000 and 22,000 mol wt components exhibited the same heterogeneity as detected in zein by starch gel electrophoresis in 6*M* urea at pH 3.5. These results show that unresolved zein is composed of several subunits displaying discrete variations in primary structure, and that it is able to exist as monomers or to associate into dimeric forms.

Zein, the major storage protein class of maize grains, is extracted by aqueous solutions of ethanol and 2-propanol. Many investigations using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) have shown that zein treated by 2-mercaptoethanol (ME) after extraction is made up of two main subunits. The heavier (H) subunit has a mean molecular weight (mol wt) between 21,000 and 25,000, whereas the lighter (L) subunit has a mol wt between 19,000 and 23,000. Two-dimensional gel electrophoresis showed that both subunits are made up of several polypeptides with different isolectric points (Hagen and Rubenstein 1980, Wilson et al 1981).

Unreduced or native zein, analyzed by SDS-PAGE (Abe et al 1981, Landry 1979, Paulis 1981, Tsai 1980) or by gel filtration (Abe et al 1981, Ganchev and Stoyanova 1972, Ivanov et al 1976, Landry 1965, Landry and Sallantin 1978) appears to contain monomeric, dimeric, and oligomeric proteins. The variable complexity of these sets and their relative percentages suggest that many zein polypeptides differ in their ability to associate with each other.

To gain additional information about the heterogeneity of unreduced zein, we used gel filtration in the presence of SDS to fractionate samples of carefully purified protein, and we characterized the chromatographic fractions by electrophoresis in the presence or absence of SDS.

MATERIALS AND METHODS

Maize grains were from the three-way hybrid INRA 260, described previously by Landry and Moureaux (1980).

Zein was isolated and purified according to the procedure developed by Landry (1979). Zein was extracted with 55% 2-propanol from meal that was free of fats and salt-soluble nitrogen, and the extract was washed with petroleum ether. Proteins in the alcohol solution were purified, first with aqueous sodium chloride and then with absolute ethanol. Protein pellets dissolved in 55% tertiary butyl alcohol were then chromatographed on Sephadex LH20 equilibrated with the same solvent. Zein was recovered by direct lyophilization of the aqueous tertiary butyl alcohol chromatographic fractions.

The purified zein sample contained 15.9% N (dry basis) and was entirely soluble in 1.5% SDS at a protein concentration of 2%. Its behavior in SDS-PAGE and in starch-gel electrophoresis at pH 3.5, its amino acid composition, and the quality of its N-terminal residues were reported previously (Landry 1979).

Gel filtration chromatography was conducted on a bed of Ultrogel Ac44 (LKB). Ultrogel was equilibrated in 1.5% SDS (w/w) for 1 hr and packed in a 2.15 × 120-cm column. After settling (3 hr), the gel bed (105 cm high) was washed with 1.5% SDS for 20 hr. Protein samples (20 mg/ml) were dissolved in 1.5% SDS by stirring at room temperature.

SDS-PAGE of unreduced or reduced (24 hr, 1 μl ME/1 mg protein) chromatographic fractions was performed in 12.5% gels in the presence of 0.1% SDS buffered with 375 m*M* Tris and 0.06*N* HCl (pH 8.9), whereas starch gel electrophoresis was performed in 12% gels in the presence of 6*M* urea and 1.5 m*M* aluminum lactate at pH 3.5 (Landry 1979). For the quantitative removal of SDS, fractions for urea starch gel electrophoresis were dialyzed against a 20-fold volume of 70% ethanol that was changed three times in six days.

RESULTS

When chromatographed on Ultrogel Ac44 in the presence of 1.5% SDS (Fig. 1), unreduced zein was resolved into one minor fraction, A, and two major ones, D and M, corresponding to

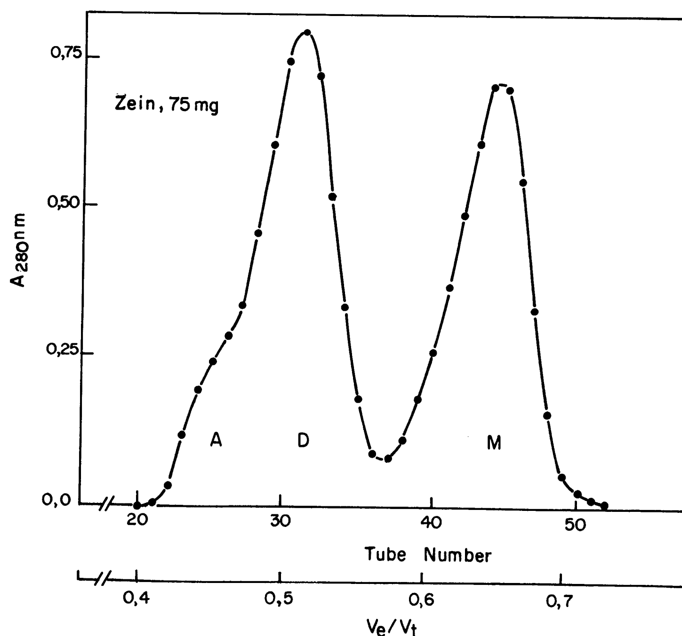

Fig. 1. Gel filtration of 75 mg of unreduced zein on an Ultrogel Ac44 column (2.15 × 120 cm). A = Minor fraction, aggregated form; D and M = major fractions, dimeric and monomeric forms, respectively.

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aggregated, dimeric, and monomeric forms, respectively. Fraction M represented 45% of the absorbance of all protein fractions at 280 nm.

SDS-PAGE analysis of the resulting chromatographic fractions is shown in Fig. 2. Fractions from peak D (tubes 30 and 36) contained two subsets of electrophoretic components migrating near ovalbumin and having 45,000 mol wt. The subset of slow-moving electrophoretic components (tube 30) eluted in a large zone, overlapping chromatographic fractions A (not shown) and M (tubes 38 and 39). The subset of fast-moving electrophoretic components eluted in a narrow zone in the descending portion of chromatographic peak D (tubes 36 and 37). The ascending portion of peak M (tubes 41 and 43) was a mixture of 24,000 and 22,000 mol wt subunits. The descending portion of peak M consisted of 22,000 mol wt subunits and comprised fractions (tubes 45–50) representing 22% of the total protein absorbance at 280 nm. Finally, fractions eluted between peaks D and M (tubes 36–39) contained slower migrating components of peak D and the 22,000 and 24,000 mol wt subunits of peak M.

Starch gel electrophoresis of chromatographic fractions shown in Fig. 1, the bulk fraction M (isolated from gel filtration of zein on hydroxypropylated Sephadex G100 equilibrated in aqueous ethanol) (Landry and Sallantin 1978), and total zein are shown in Fig. 3. The similarity of the patterns for zein and M, whether untreated by SDS (cZ and cM) or treated by SDS and then dialyzed (dZ and dM), indicated that SDS is completely removed from its complex with proteins after dialysis against aqueous ethanol. Fractions of peak D (tubes 28, 30, and 33) separated into a series of regularly spaced bands. The 22,000 mol wt subunits in fractions of the descending portion of peak M (tubes 45 and 47) exhibited bands with the same mobilities as the major band in total M and zein. Proteins eluted between peaks D and M (tubes 36–39) had a band pattern similar to components of peak M (tubes 45–47) and contained two additional bands: the slowest migrating band, and the slowest band in the fastest migrating triplet.

A second chromatography on the same bed of Ultrogel Ac44 was performed to fractionate a larger sample of unreduced zein (Fig. 4) and to recover more concentrated fractions. The same fractions A, D, and M were isolated, but peaks D and M were less well resolved than in the first chromatography, probably because of column overload.

Upon analysis by SDS-PAGE (Fig. 5A) fractions from this second chromatography (Fig. 4) were similar to those from the first chromatography (Fig. 2). Some trimers were evident in peak A (tube 32).

The same chromatographic fractions were also analyzed by SDS-PAGE after reduction of disulfide bands (Fig. 5B). All fractions, except those eluting in the descending portion of peak M, contained both 24,000 and 22,000 mol wt subunits, but the relative abundance of the 24,000 mol wt subunits decreased progressively from A to M. Two very faint bands migrating slightly slower or faster with cytochrome C and having mol wt of 14,000 and 10,000 were also detected in the fractions from the descending portions of peak D (tubes 41, 44, and 45).

DISCUSSION

Filtration of unreduced zein on Ultrogel Ac44 in the presence of SDS demonstrates the existence of three fractions consisting of monomeric, dimeric, and oligomeric species, and leads to a fast (about 24 hr) and efficient separation of dimers and monomers. Electrophoretic analysis of chromatographic fractions shows that

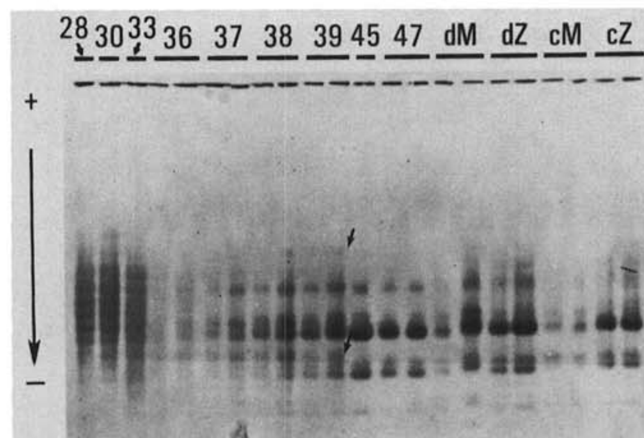

Fig. 3. Starch gel electrophoresis (6*M* urea, pH 3.5) of chromatographic fractions from Fig. 1, along with patterns for zein (Z) and total fraction M (M). c = Control; d = solubilized in the presence of SDS and dialyzed against aqueous ethanol. Sample load (μg per 0.7 × 0.5 cm paper wick): patterns 28, 30, 33:400; 36–39:50 and 100; 45:15; 47:15 and 30; dM and cM:30 and 60; dZ and cZ:100 and 200. Arrows indicate migration of additional bands found in patterns 36–39 with respect to those designated 45 and 47.

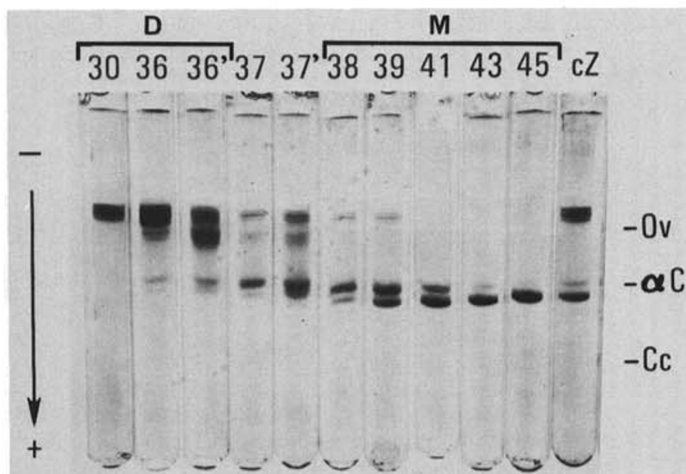

Fig. 2. SDS-PAGE of chromatographic fractions from Fig. 1. Patterns 36′ and 37′ are duplicates of those designated 36 and 37, run at twice the sample load. cZ = Control unreduced zein. Horizontal bars indicate migration of standard proteins (not shown): ovalbumin (Ov), mol wt 45,000; α-chymotrypsinogen (αC), mol wt 25,700; and cytochrome C (Cc), mol wt 12,500.

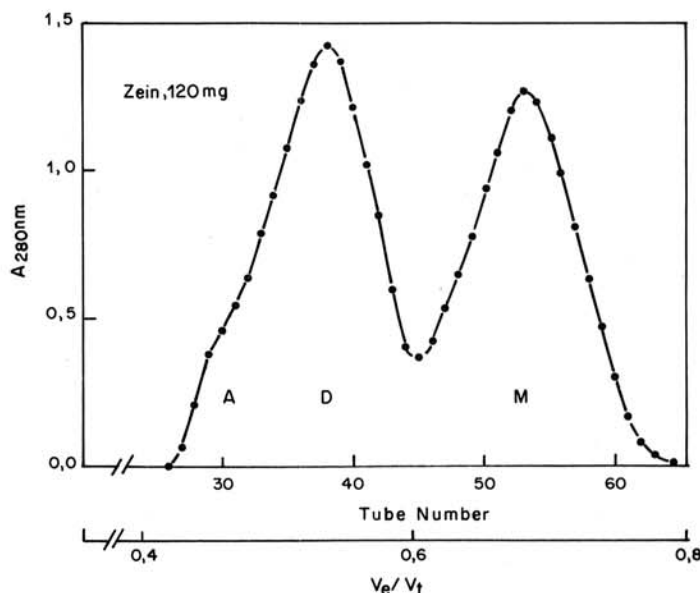

Fig. 4. Gel filtration of 120 mg of unreduced zein on an Ultrogel Ac44 column (2.15 × 120 cm). **A** = Minor fractions aggregated form; **D** and **M** = major fractions, dimeric and monomeric forms, respectively.

22,000 mol wt subunits, nearly free of the 24,000 mol wt subunits, can be isolated on the basis of their late elution from gel, agreeing with the results of Ganchev et al (1979) and of Abe et al (1981). Electrophoresis at pH 3.5 (Fig. 3) shows that these subunits, because they have the same size, contain variable amounts of charged residues and consequently possess different primary structures.

We were unable to isolate 24,000 mol wt subunits from 22,000 mol wt ones. Similarly, Ganchev et al (1979) failed to separate them by gel filtration on Sephadex G200 equilibrated in 35% acetic acid. Abe et al (1981), however, isolated 24,000 mol wt subunits in the earlier fractions obtained by chromatography of very low amounts of reduced zein (2 mg) on Sephacryl (1.8 × 142 cm) in the presence of 0.1% SDS. Comparison of electrophoretic behavior of M and 22,000 mol wt subunits at pH 3.5 suggests that 24,000 mol wt subunits exhibit charge heterogeneity similar to the 22,000 mol wt subunits. However, their primary structures differ because their tendency to associate into dimers is not the same.

The association of the 22,000 mol wt (L) and 24,000 mol wt (H) zein polypeptides into dimers could lead to formation of two forms of homodimers (HH and LL) and one form of heterodimer (HL), resulting in three bands with mobilities similar to ovalbumin in SDS-PAGE. Indeed, bands apparently representing these three dimeric forms are detected in patterns of total zein and in fractions of peak D, where they represent the set of slowest migrating entities (Fig. 2, tube 30). Similar observations were reported by Abe et al (1981).

SDS-PAGE analysis of reduced chromatographic fractions from the descending portion of peak D showed the existence of low mol wt components, which may be identical to G_1-glutelin polypeptides extracted in bulk after zein by aqueous alcohols in the presence of a reducing agent (Gianazza et al 1976, Paulis and Wall 1977) (Fig. 5B, tubes 41–45). These polypeptides would associate with zein subunits to form heterodimers constituting the fastest-migrating entities of peak D and probably represented by additional bands detected in starch gel electrophoretic patterns of

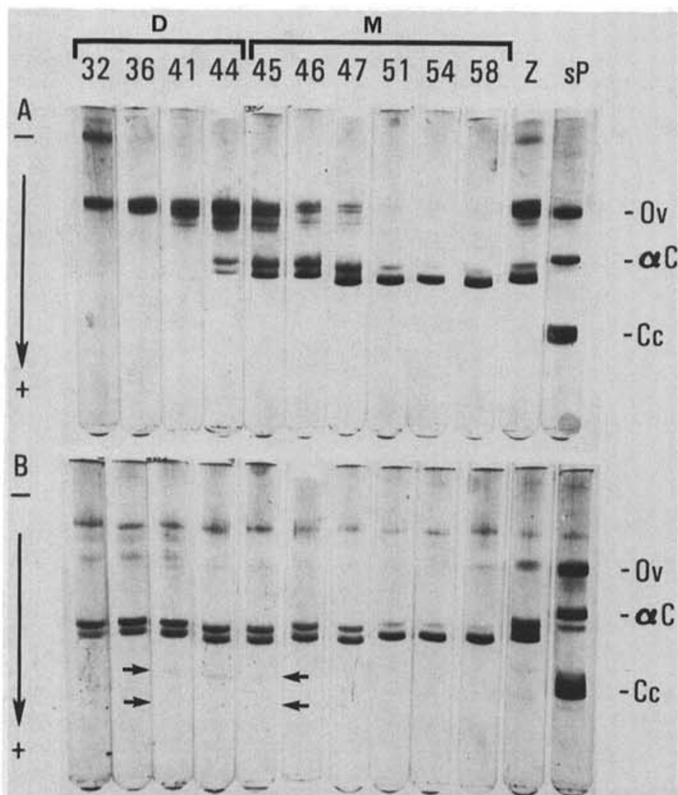

Fig. 5. SDS-PAGE of chromatographic fractions from Fig. 4. **A** = Unreduced samples; **B** = reduced (2-mercaptoethanol) samples. Standard proteins (sP) as in Fig. 2.

proteins eluted at the end of peak D.

Zein, therefore, appears to consist of several subunits that display discrete variations in primary structure and are able to exist as monomers or to associate into stable dimeric and oligomeric forms.

The relative amounts of zein monomers, dimers, and oligomers depend on operating conditions, as suggested by studies reporting the gel chromatographic behavior of unreduced zein. In an earlier investigation (Landry 1965), a zein sample purified by a procedure similar to that described here, was resolved into three minor fractions A, B, and E, and into two major ones, C and D, by gel chromatography on Sephadex G200 equilibrated with 8*M* urea. The similarity of this chromatogram to those presented in Figs. 3 and 4 suggests that fractions A plus B, C, and D, respectively, correspond to fractions A, D, and M, and that gel filtration of zein is largely independent of the medium used. In contrast, Ganchev et al (1972) submitted unreduced zein to gel filtration on Sephadex G200 in 8*M* urea and obtained three fractions (A, B, and C) having apparent mol wt of 180,000, 100,000, and 52,000, respectively. Gel chromatography of reduced zein caused Ivanov et al (1976) to conclude that A and B represent oligomers and dimers, respectively, of fraction C, which contains the lighter subunit resolved by SDS-PAGE and represents 20% of total zein (Ganchev et al 1979). Similar observations were reported by Reva et al (1978) concerning partially purified zein; pore-gradient PAGE in the presence of urea and acetic acid and gel filtration on Sepharose CL-6B in the presence of 8*M* urea showed that twelve electrophoretic and five chromatographic fractions have apparent mol wt ranging from 40,000 to 280,000. The same zein sample, examined by SDS-pore-gradient electrophoresis, was found to consist of two main subunits of 20,200 and 24,400 mol wt and six minor reduced components with mol wt ranging from 12,400 to 59,900 (Rewa and Brückner 1979). More recently, Paulis (1981) also reported a predominance of high mol wt components for unreduced zein and its 95% ethanol-soluble fractions, which is close to the zein preparation studied here, and confirmed the existence of disulfide-linked oligomers on the basis of their dissociation following treatment with 2-ME.

These observations show that SDS solutions, compared to 8*M* urea, promote the dissociation of oligomers, which are present only in unreduced zein. They suggest that the oligomer structures are stabilized through intrachain or perhaps interchain disulfide links and through noncovalent bonds—mainly hydrophobic interactions—which is consistent with the conclusions of Rewa and Brückner (1979).

The previous reports of Williams and Watson (1938) should also be noted. These authors isolated three zein fractions by successively reducing the ethanol content of solution at 49, 45, and 35%; their mol wt, as determined from sedimentation velocity and diffusion data, and their percentages were: 80,000 (25%), 40,000 (50%), and 22,000 (25%), which represented a good estimate of size heterogeneity of unreduced zein.

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