ZEIN: A HETEROGENEOUS PROTEIN CONTAINING DISULFIDE-LINKED AGGREGATES[1]

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ABSTRACT

Agar and starch gel electrophoresis showed that zein is heterogeneous and also that this prolamine of corn, as it exists naturally, contains disulfide-linked aggregates. Native zein exhibited several mobile components and a fraction that did not migrate into the gel upon electrophoresis. All components were mobile following reduction of disulfide bonds. Reduction of native zein also lowered the weight-average molecular weight from 44,000 to 21,000. The mobile fraction was identical to alpha zein, a fraction soluble in 95% ethanol. The immobile fraction resembled beta zein, which is insoluble in 95% ethanol.

The molecular structure of corn zein protein has eluded characterization despite extensive investigation by many workers. In applications of previously existing techniques, unfavorable conditions have been used because of the limited solubility of this protein. Now gel electrophoresis conclusively demonstrates the electrophoretic heterogeneity of zein. Also, the presence of intermolecular disulfide bonds in one or more zein components is established.

Zein is defined as the protein extractable from corn with aqueous alcohols.[3] It is soluble in water at high concentrations of urea, at pH 11 and above, or in the presence of anionic detergents. These extreme conditions for solubility are probably attributable to the amino acid composition of the protein, which is rich in glutamine, leucine, and proline residues but deficient in basic or acidic amino acids. More detailed information on composition and properties of zein is available in the comprehensive review of Mossé (1).

One early method for studying zein, moving boundary electrophoresis, indicated that the protein was heterogeneous (2,3,4,5,6), but firm conclusions could not be reached then because of anomalies caused by the buffer systems or solvents employed. In other studies (3,4,5) the use of high pH systems may have reduced the electrostatic differences between protein species by causing tyrosine to ionize or by chemically altering the protein, for example, disrupting disulfide

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[3]Such zein differs from commercially prepared zeins which have been isolated in such a manner as to cause chemical modification in them. These modifications will be discussed in a forthcoming publication.

bonds. Since anionic detergents combine with charged groups of the protein (4), they alter the total net charge on the molecules. Ethanol-water solutions promote association-dissociation complexes of zein molecules (2), and high concentrations of urea in moving boundary cells cause turbulence and render the electrophoretic pattern indistinct.

Another method for study of zein composition has been solvent fractionation, which has been widely used as a tool to show that zein consists of more than a single component. However, the fractions obtained generally were poorly defined and not readily duplicated, even though differences in molecular weights were demonstrated by some workers (6,7,8,9,10,11). Watson, Arrhenius, and Williams (6) employed various concentrations of ethanol in water to separate zein into two or more components; Gortner and MacDonald (7), aqueous methyl cellosolve; and Donard and Labbé (8,9), amyl alcohol. Two zein fractions, called alpha zein and beta zein, were described by McKinney (12). Alpha zein is defined as that prolamine of corn gluten which is soluble in 95% ethanol and represents about four-fifths of the total prolamine. It corresponds closely to the common zein of commerce before 1957. Beta zein is that which is soluble in 60% ethanol but is insoluble in 95% ethanol. Because it is relatively unstable in solution, it was not included in commercial zein preparations. Preparations of zein obtained by extraction with intermediate concentrations of alcohol should contain both types.

Gel electrophoresis proved advantageous in studying zein because it permits use of buffers containing high concentrations of urea and pH levels that are best for effective resolution of components. In addition, proteins of different molecular weights may exhibit variations in mobility due to the sieving action of the gel.

Disulfide linkages in wheat gluten proteins were shown by Nielsen *et al.* (13) to account for the high molecular weight of some components in which lower-molecular-weight polypeptides were cross-linked. Disulfide reduction studies, therefore, were carried out on zein and zein fractions, and the products were investigated by gel electrophoresis and ultracentrifugal methods. These studies revealed that intermolecular disulfide bonds are important in the structure of some fractions of the zein protein complex.

Materials and Methods

Analytical Methods. Nitrogen was determined by the Kjeldahl method with mercuric oxide as catalyst. Sulfur was determined by the Carius gravimetric method as described by Steyermark (14).

Preparation of Zein. Zein was prepared from partially defatted (15) ground corn of two varieties (Dyar 444 and Schwenk U.S. 13). The corn meals were extracted with either 60 or 70% (vol./vol.) aqueous ethanol at room temperature for 5 hr. The extracts were then dialyzed against distilled water and the precipitated protein was centrifuged and lyophilized (3). The protein prepared in this manner assayed between 14 and 15% N and 0.5 to 0.6% S. It was a yellow granular solid, nearly all of which could be redissolved in the original extraction solvent.

Gel Electrophoresis. Starch gels were prepared as described by Smithies (16). The buffer at pH 3.5 was aluminum lactate-lactic acid, nominal ionic strength 0.05 (17), containing 8*M* urea. Sixteen grams of starch[4] was added to 100 ml. of the 8*M* urea buffer solution in a Waring Blendor to break down the initial gel that forms upon addition of the starch to the buffer solution. The slurry was then heated on a steam bath until it cleared and the solution could be poured. Twenty-four hours were required for a firm set of the gel. Electrophoresis was carried out as described by Woychik *et al.* (18) for a period of 24 hr. with a field strength of 20 v./cm. in a Reco, Model E-800-2, electrophoresis migration chamber. The 8*M* urea-buffer solution was used in the buffer wells, and filter paper wicks served for contact. The gels were then sliced transversely and stained for 5 min. with 2% nigrosine dye in water. The excess dye was eluted with several changes of water.

Agar gels were prepared by slurrying 3 g. of washed agar with 100 ml. of the urea-buffer solution employed for starch gels, heating until dissolved, and placing the resulting clear solution in trays to freeze. Freezing of the agar-urea-buffer solutions was essential for gel formation. Upon thawing, the gels were firm and transparent. Electrophoresis was performed in the same manner as described for the starch gels, but the gels were not sliced before being stained.

Preparation of Alpha and Beta Zein Fractions. Approximately 1 g. of whole zein, which assayed 14.4% N and 0.59% S (d.b.), was stirred with 100 ml. of 95% ethanol for 3 hr. at room temperature and centrifuged. The insoluble protein was washed with several 10-ml. portions of fresh ethanol until the supernatant was free of color. The 95%-ethanol-soluble protein, alpha zein, was precipitated from the combined supernatants by dialysis against water, and the precipitate was recovered and lyophilized. This material assayed 14.6% N and 0.34% S (d.b.).

[4] Starch Hydrolyzed, Connaught Medical Research Laboratories, Toronto, Canada. Mention of firm names or trade products is for identification only and does not imply endorsement by the U.S. Department of Agriculture.

The centrifuged 95%-ethanol-insoluble material, beta zein, was stored in a freezer. A portion of the frozen protein was dried with acetone and ether before nitrogen and sulfur analysis. It assayed 14.3% N and 0.93% S (d.b.).

Cleavage of Disulfide Bonds. Zein was reduced with cupric sulfite solution as described by Mertz and Bressani (19), with a reaction time of 4 hr. The protein solution was dialyzed at 4°C. for 3 days against 0.02*M* HCl and for 3 days against distilled water, and finally lyophilized.

For reduction with mercaptoethanol, zein was dissolved in 8*M* urea-0.01*M* phosphate, pH 8.0, containing 50 molar excess of mercaptoethanol (20), assuming 1 mole cystine per 25,000 g. zein. The reduction was allowed to proceed for 4 hr. at 25°C. Thiol groups were alkylated by the addition of a twofold excess of acrylonitrile (21). After alkylation for 4 hr., the protein solution was dialyzed until free of urea and alkylating agent and then lyophilized.

Zein was oxidized with performic acid at 25°C. following the procedure described by Hirs (22) for cleaving disulfide bonds. The oxidized products were precipitated by the addition of water and either frozen or lyophilized.

Molecular Weight Determination. Approach to equilibrium sedimentation patterns were obtained with a double sector cell in a Spinco Model E ultracentrifuge at 19,160 r.p.m. The molecular weights were calculated according to the Archibald method (23), using a measured partial specific volume for whole zein. The partial specific volume was calculated from density measurements made in 8*M* urea-aluminum lactate buffer, nominal ionic strength 0.05, pH 3.5, at 24.9°C., after the protein solutions had been kept at room temperature for 48 hr. Two determinations averaged 0.758 ± 0.001 for the partial specific volume.

Results and Discussion

Agar and Starch Gel Electrophoresis of Zein. The electrophoretic patterns of whole native zein, in both agar and starch gels, showed several mobile components plus material that did not migrate. Zein exhibited a different pattern on the two types of gels, as seen in Figs. 1, a, and 2, a. The two major components were much faster migrating in agar gels than in starch gels. In addition, a greater proportion of the protein appeared to migrate into the agar gel, broadening the origin region and possibly contributing to the increased number of components visible. The greater mobility, as well as the increased number of components, in the agar gels is probably

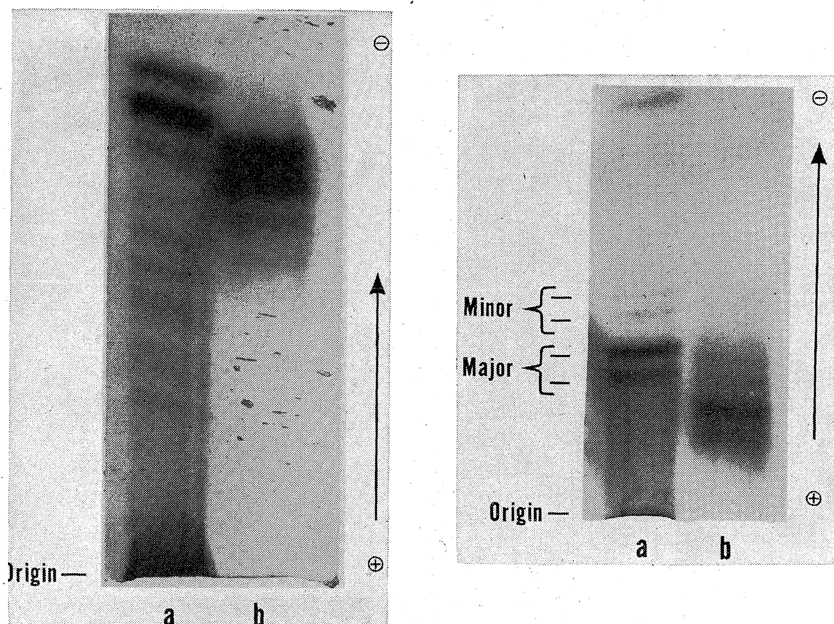

Fig. 1 (left) and Fig. 2 (right). Fig. 1. Agar gel electrophoretic pattern of native (a) and oxidized (b) zein. Fig. 2. Starch gel electrophoretic pattern of native (a) and oxidized (b) zein.

caused by the combination of two factors — electro-osmosis and gel porosity. It is well known that the highly charged agar gels have a greater electro-osmotic flow than the lesser charged starch gels; the flow would accelerate the migration of the components toward the cathode. The difference in gel porosity would be due to the difference in solids content of the two gels; agar gels are 3% agar; the starch gels are 16% starch. The more porous agar gels would undoubtedly allow larger molecules to penetrate the gel matrix.

Starch gels were used regularly for the studies because of their high quality; they were transparent and quite easily handled when they contained 8*M* urea.

The zein pattern shown on starch gel in Fig. 2, a, is typical of many assays. From the corns used, zein always presented two major bands, two minor ones, and origin material with "tailing" quite evident.

Gel Electrophoresis after Disulfide Cleavage. Information concerning the origin material present in zein was sought by cleaving disulfide bonds with performic acid. The agar gel and starch gel patterns of the oxidized zein are shown in Figs. 1, b, and 2, b, respec-

tively. Immobile origin material was absent, but two major bands and several minor ones occurred in both types of gel. The absence of immobile origin material in the oxidized zein indicates that intermolecular disulfide bonds had been broken, thereby decreasing the molecular size. While fragmentation of the molecules by peptide chain cleavage might give similar results, Hirs (22) found no evidence of such cleavage during performic acid oxidation of disulfide bonds.

Disulfide bonds also were cleaved by reducing the zein with copper sulfite. The resulting starch gel pattern (Fig. 3, c) was identical to that of the performic acid-oxidized zein (Fig. 3, b); they contained the same major bands, several minor ones, and no origin material. However, the two major components in both the oxidized and sulfite-reduced zein had lower mobility than did those in the whole native zein.

To determine whether the change in electrophoretic mobility following reduction or oxidation was primarily a result of disulfide cleavage or a change in molecular charge, zein was also reduced with mercaptoethanol and then alkylated with acrylonitrile. This alkylation reaction was shown by Weil and Seibles (21) to stabilize the structure of the reduced protein without changing the molecular charge from that of the protein before reduction. In Fig. 4, reduced-alkylated zein (b and c) is compared with unmodified whole zein (a) by starch gel electrophoresis. The reduced-alkylated zein had two major components, as well as several minor ones, and no material at the origin. The two major components in the reduced-alkylated zein pattern had nearly the same mobility as the major components in whole zein, in contrast with the considerably lower mobilities for these components

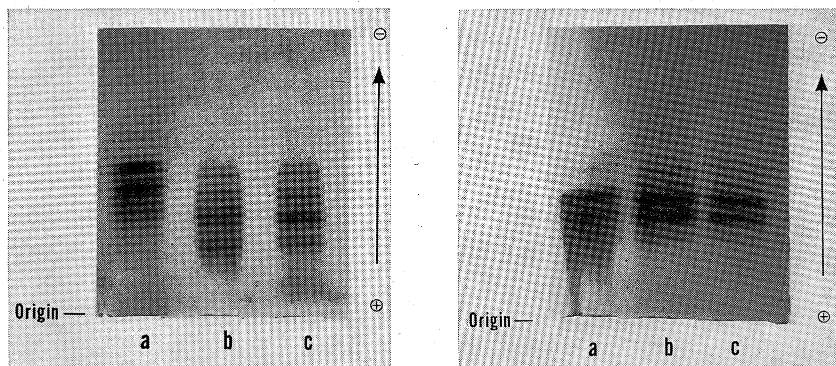

Fig. 3 (left) and Fig. 4 (right). Fig. 3. Starch gel electrophoretic pattern of native (a), oxidized (b), and sulfite-reduced (c) zein. Fig. 4. Starch gel electrophoretic pattern of native (a) and two reduced-alkylated (b, c) zeins.

in the performic acid-oxidized or the copper sulfite-reduced zein patterns. Apparently, the change in the mobilities following oxidation or sulfite reduction is the direct result of the addition of negative sulfonic or S-sulfonic acid groups to the protein. The slight decrease in mobility of the major components in reduced-alkylated zein could result from changes in the conformation of the molecules.

Although the patterns of the oxidized and the sulfite-reduced zeins differed from those of the reduced-alkylated zein in respect to mobility, the number of components and the apparent concentration of each were virtually the same. This similarity is consistent with the conclusion that cleavage of disulfide bonds yields individual polypeptide species which are able to migrate into the gel.

Electrophoresis of Alpha and Beta Zeins. Alpha zein and beta zein fractions gave starch gel electrophoresis patterns of which Fig. 5

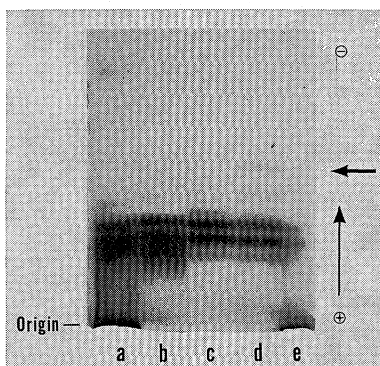

Fig. 5. Starch gel electrophoretic pattern of native (a), alpha (b), reduced-alkylated alpha (c), reduced-alkylated beta (d), and beta (e) zeins.

is typical. Alpha zein (Fig. 5, b) was completely mobile and its gel components were identical with the mobile components of whole zein (Fig. 5, a). In contrast, beta zein (Fig. 5, e) did not migrate from the origin and was analogous to the immobile fraction previously demonstrated in whole zein. Since the origin material of whole zein had proved to be a disulfide-linked polymer, beta zein was reduced with mercaptoethanol and alklyated. Alpha zein was treated in the same manner for comparison. Shown in Fig. 5 are the gel patterns of the reduced-alkylated alpha (Fig. 5, c) and beta (Fig. 5, d) fractions.

Reduced-alkylated alpha zein and reduced-alkylated beta zein contain nearly all the components present in reduced-alkylated whole zein. The major components of each appear to be in the same con-

centration relative to the minor components. The one exception is the fastest-moving component in reduced-alkylated beta zein (Fig. 5, d). This minor band seems to be in a slightly higher concentration in beta zein than in alpha zein.

Beta zein and the immobile origin material present in native zein apparently are identical, whereas alpha zein corresponds to the native mobile components. It has also been demonstrated that beta zein is a disulfide-linked aggregate normally found in native zein and, presumably, is a relatively high molecular-weight material.

Molecular-Weight Data. Whole and reduced-alkylated zeins were measured in the ultracentrifuge in the same 8*M* urea aluminum-lactate buffer (pH 3.5) used as the solvent in the gel electrophoresis studies. Qualitatively, the patterns indicated heterogeneity; only one component peak appeared but it was slightly skewed toward the high-molecular-weight side in the whole zein. The pattern for reduced zein was less skewed, indicative of reductive fragmentation of at least much of the highest molecular-weight material. The molecular weights were calculated by Archibald's method (23), which gives a weight-average molecular weight. At two concentrations, 0.3 and 0.6% protein, the whole zein exhibited a weight-average molecular weight of 44,000. At the same two concentrations, the reduced-alkylated zein had a molecular weight of approximately 21,000. The molecular weight after disulfide cleavage is consistent with the minimum unit weight of 19,600 recently reported by Krull *et al.* (24) based on amino nitrogen analysis of N-terminal groups in a dimethyl sulfoxide solution of whole zein.

Some of these polypeptide units, then, must be combined through disulfide bonds to give a mixture of molecules with the higher weight-average molecular weight of 44,000. The presence of these larger, cross-linked molecules also was shown by the gel electrophoretic patterns.

Conclusions

The demonstration that zein, as extracted directly from corn, consists of several protein species including at least one that is a disulfide-linked aggregate of smaller components may help to resolve many of the contradictory observations reported in the past. Differences in composition and molecular weight of this protein reported by many workers may result from variations in extraction and treatment procedures employed to obtain the protein. Comparison of data for native zein with that for commercial zein can be expected to show discrepancies because of the initial fractionation and chemical modi-

fication inherent in the commercial preparations of zein. It is also evident that many physical measurements in the protein presupposing a homogeneous system are not valid. Studies of molecular asymmetry, if performed on alkali-treated or reduced zein, may give evidence of molecular shapes different from that of the native protein due to disruption of disulfide bonds.

The elucidation of the heterogeneity of zein and the participation of disulfide bonds in its structure open additional avenues of investigation of this important protein. The methods and techniques reported here should be useful tools for evaluating the characteristics of the protein preliminary to any investigation of composition or structure. Such aids should also be valuable in further fractionation studies, since they permit reliable evaluation of the homogeneity of the zein fractions.

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Literature Cited

1. MOSSÉ, J. Monographie sur une protéine du mais: La Zéine. Ann. Physiol. Vegetale **3** (2): 105–139 (1961).
2. SCALLET, B. L. Zein solutions as association-dissociation systems. J. Am. Chem. Soc. **69:** 1602–1608 (1947).
3. CRAINE, E. M., FREIMUTH, DIANE V., BOUNDY, JOYCE A., and DIMLER, R. J. Preparation of purified zein by adsorption-desorption. Cereal Chem. **38:** 399–407 (1961).
4. FOSTER, J. F., YANG, J. T., and YUI, N. H. Extraction and electrophoretic analysis of the proteins of corn. Cereal Chem. **27:** 477–487 (1950).
5. MERTZ, E. T., LLOYD, N. E., and BRESSANI, R. Studies on corn proteins. II. Electrophoretic analysis of germ and endosperm extracts. Cereal Chem. **35:** 146–155 (1958).
6. WATSON, C. C., ARRHENIUS, S., and WILLIAMS, J. W. Physical chemistry of zein. Nature **137:** 322–323 (1936).
7. GORTNER, R. A., and MACDONALD, R. T. Studies on the fractionation of zein. Cereal Chem. **21:** 324–333 (1944).
8. DONARD, E., and LABBÉ, H. Sur une matière albuminoide extraite du grain de mais. Compt. Rend. **135:** 744–746 (1902).
9. DONARD E., and LABBÉ, H. Les matières albuminoides du grain de mais. Compt. Rend. **137:** 264–266 (1903).
10. LINDET, M., and AMMAN, L. Sur le pouvoir rotatoire des protéines extraites des farines de céréales par l'alcool aqueux. Bull. Soc. Chim. **1** (4): 968–974 (1907).
11. ELLIOTT, M. A., and WILLIAMS, J. W. The dielectric behavior of solutions of the protein zein. J. Am. Chem. Soc. **61:** 718–725 (1939).
12. MCKINNEY, L. L. Zein. *In* Encyclopedia of chemistry (supplement), ed. by G. L. Clark, pp. 319–320. Reinhold: New York (1958).
13. NIELSEN, H. C., BABCOCK, G. E., and SENTI, F. R. Molecular weight studies on glutenin before and after disulfide-bond splitting. Arch. Biochem. Biophys. **96:** 252–258 (1962).
14. STEYERMARK, A. Alternate gravimetric Carius procedure. *In* Quantitative organic microanalysis (2nd ed.), p. 290. Academic Press: New York and London (1961).

15. CRAINE, E. M., and FAHRENHOLTZ, K. E. The proteins in water extracts of corn. Cereal Chem. **35:** 245–259 (1958).
16. SMITHIES, O. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. Biochem. J. **61:** 629–641 (1955).
17. JONES, R. W., TAYLOR, N. W., and SENTI, F. R. Electrophoresis and fractionation of wheat gluten. Arch. Biochem. Biophys. **84:** 363–376 (1959).
18. WOYCHIK, J. H., BOUNDY, JOYCE A., and DIMLER, R. J. Starch gel electrophoresis of wheat gluten proteins with concentrated urea. Arch. Biochem. Biophys. **94:** 477–482 (1961).
19. MERTZ, E. T., and BRESSANI, R. Studies on corn proteins. I. A new method of extraction. Cereal Chem. **34:** 63–69 (1957).
20. SELA, M., WHITE, F. H., JR., and ANFINSEN, C. B. The reductive cleavage of disulfide bonds and its application to problems of protein structure. Biochim. Biophys. Acta **31:** 417–426 (1959).
21. WEIL, L., and SEIBLES, T. S. Reaction of reduced disulfide bonds in α-lactalbumin and β-lactalbumin with acrylonitrile. Arch. Biochem. Biophys. **95:** 470–473 (1961).
22. HIRS, C. H. W. The oxidation of ribonuclease with performic acid. J. Biol. Chem. **219:** 611–621 (1956).
23. ARCHIBALD, W. J. A demonstration of some new methods of determining molecular weights from the data of the ultracentrifuge. J. Phys. Colloid Chem. **51:** 1204 (1947).
24. KRULL, L. H., WALL, J. S., and DIMLER, R. J. Dimethyl sulfoxide as solvent for amino nitrogen determination on cereal proteins by the Van Slyke manometric method. Anal. Biochem. **6:** 375–380 (1963).