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Multiple Zeins from Maize Endosperms Characterized by Reversed-Phase High Performance Liquid Chromatography

Curtis M. Wilson

U.S. Department of Agriculture, Agricultural Research Service, Northern Regional Research Center, Plant Polymer Research, Peoria, Illinois 61604

ABSTRACT

The major storage proteins of maize (*Zea mays* L.) endosperm are located in protein bodies, and may be separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into two major classes and four minor classes of polypeptides. The two major classes (commonly known as zeins) have been separated previously into a large number of components by isoelectric focusing (IEF). Reversed-phase high performance liquid chromatography (HPLC) further separated the major classes into additional components, and gave distinctive peaks for each minor zein class. Some IEF bands produced two or more HPLC fractions, while some HPLC fractions produced two or more IEF bands. Apparently identical IEF bands from different inbreds may appear in different fractions after HPLC. Thus the total number of zeins revealed by separations based on apparent size (SDS-PAGE), net charge (IEF), and hydrophobicity (HPLC) is very large. Different laboratories have developed diverse nomenclatures which cause much confusion. A key is presented to provide a flexible and expandable nomenclature for this complex group of proteins.

Zein, the major storage protein in maize endosperm, was initially characterized as a prolamin by its solubility in aqueous alcohol solutions and its high content of proline and amide nitrogen (13). The Osborne extraction system has long been used to define seed proteins, but solubility and extractability are difficult to apply reproducibly. Reducing agents (11) and reducing agents plus sodium acetate (16) in alcoholic solutions extract additional proteins. These proteins resemble classical zein in having no lysine or tryptophan, are relatively high in proline and glutamine, and are located in protein bodies (24, 29). It is convenient to consider these proteins together, but different workers have set up different nomenclatures. Osborne glutelin was subdivided to give an ASG[1] fraction, which was further divided into wi-ASG and ws-ASG fractions (16). Some felt that the term glutelin was no longer useful in describing these alcohol-soluble proteins, which were placed into an enlarged zein family of proteins (20, 29). These proteins are also called zein associated proteins or ZAP (19).

[1] Abbreviations: ASG, alcohol-soluble glutelin (with wi-, water-insoluble and ws-, water-soluble fractions); ACN, acetonitrile; IEF, isoelectric focusing; ME, 2-mercaptoethanol; PM, 55% (v/v) isopropanol with 5% (v/v) ME added; PMA, PM with 0.5% (w/v) sodium acetate added; R_m, relative mobility; RP, reversed-phase; SPE, 0.5 M NaCl, 0.05 M phosphate buffer, pH 7.8, 0.01 M EDTA.

I use the following definition. Zeins are alcohol-soluble proteins that occur principally in protein bodies of maize endosperm and which may or may not require reduction before extraction. Classes of zeins can be distinguished primarily by R_m on SDS-PAGE and secondarily by solubility, amino acid composition (little or no lysine and tryptophan, relatively high glutamine and proline, and specifically high concentrations of certain amino acids such as methionine, cysteine, or histidine) and other properties. Six classes of zein proteins have been separated by SDS-PAGE (28), have been located in protein bodies (24), and have been characterized by molecular genetic techniques (9, 10, 19, 21). These classes are here called A and B zeins (equivalent to α or 22 and 19 kD zeins, respectively), C zein (β or 14–15 kD zein), D zein (δ or 10 kD zein), E (γ or 27 kD zein) and F (γ or 16 kD zein) (23). I have been working to provide individual identities to zein proteins through characterizing their protein properties.

RP-HPLC separates maize alcohol-soluble proteins into four groups. Peaks 1, 2, and 3 are ASGs and the multiple peak area 4 contains the major zeins, plus the "10,000 mol wt band" (14). Previous serial analysis (IEF followed by SDS-PAGE) of zeins from 18 inbreds revealed a total of 70 different zeins, while 23 different AB zeins were found in just four inbreds (28). HPLC alone is shown here to separate 34 different AB zeins from the same four inbreds, while triple serial analysis with IEF, SDS-PAGE, and HPLC separated 40 zeins. When coupled with an efficient alcoholic extraction, HPLC is a rapid and convenient method for quantitative analysis of all six classes of zein and for qualitative analysis of the many AB zeins.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L.) seeds, produced by controlled pollinations, were obtained from the Agronomy Department, University of Illinois, Urbana, IL, or were grown locally. Mature ears were dried at 40 to 45°C, and the kernels were stored at 5°C. Single seeds were sampled using a small power drill to remove endosperm tissue as a fine powder. The seed may then be saved for planting. The endosperm was extracted directly by PM or PMA solvents for at least 2 h or overnight at room temperature with occasional mixing. Sodium acetate improves the extraction of alcohol soluble proteins from maize endosperm (16). A solvent:endosperm ratio of 5:1 (v/w) was used for IEF and preparative HPLC assays and a 40:1

ratio for analytical HPLC. Recently it was found that sodium acetate in PMA does not interfere with IEF. For SDS-PAGE, 25 μL of an IEF extract were added to 1 mL of SDS sample buffer. Samples for preparative HPLC were preextracted with cold SPE buffer to reduce the amount of materials which might plug the column when large samples are applied. The SPE-extracted endosperm tissue was washed briefly with water, then acetone, and air dried.

Electrophoresis

IEF (2 μL, or about 20 μg zein/lane) was run in 0.75 mm thick gels of 1% agarose with 1.6% pH 5 to 8 or 6 to 8 Ampholine[2] plus 0.4% pH 3.5 to 9.5 Ampholine, 5 M urea, and 2 mM DTT (27). SDS-PAGE was performed on slab gels with a special gel as described elsewhere (28) to obtain separation of the AB zeins into subclasses. An estimated 3 to 12 μg of total zein were applied to reveal AB zeins only, and 25 to 50 μg for all zeins. Gels were stained with Coomassie blue R and destained with a TCA solution to avoid destaining of zeins by an alcoholic solution (28), or were stained with colloidal Coomassie blue G (12).

RP-HPLC

The RP-HPLC system used has been described elsewhere (14). Changes include use of Vydak C_{18}, 5 μ, 300 Å columns, 250 × 4.6 mm for analytical HPLC and 250 × 10 mm for preparative HPLC. The columns were equilibrated with starting buffer and were held at 55°C. Several segmented gradients of increasing ACN with 0.10% TFA were tested, but most work was done with two. If only AB zeins were to be assayed, the starting buffer was 40% ACN, increasing at 2%/min for 5 min, 0.143 %/min for 35 min, and 0.627 %/min for 15 min. Zeins A, B, C, D, E, and F were assayed starting at 38.3% ACN, increasing at 0.502 %/min for 13.6 min, at 1.95 %/min for 2.8 min, at 0.100 %/min for 13 min, at 0.1986 %/min for 18 min, and at 2.48 %/min for 3.8 min, ending at 64.92%.

Gradient slopes were selected to give good separation of zeins with a minimum of time between groups of zeins. Different gradients produced different spacing of zeins, but usually did not affect the order. Flow rates were 1 mL/min for the analytical column and 3 mL/min for the preparative column. The eluate was monitored at 210 nm and 280 nm for the analytical column and at 280 nm for the preparative column. The analytical column was usually loaded with about 25 μg of total zein in 20 μL. Preparative columns were loaded with 1 to 1.5 mg zein in 100 to 150 μL, extracted with PMA at a 5:1 ratio from 20 to 30 mg of SPE-extracted endosperm. The columns were protected with a small Synchropak C_{18} guard column.

HPLC data was stored in a ModComp computer for subsequent plotting and integration, either automatically or after manual definition of peaks. For qualitative identification of zeins, the plotted data are normalized so that the highest peak in each chromatogram has the same height.

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Serial Analysis

Zeins identified by one analysis were further identified by collecting individual fractions and running one or more additional assays. This is termed "serial analysis" to distinguish it from the more common two-dimensional systems, where all fractions of a sample are simultaneously separated by the second system. Three types of serial analyses were done. In one, stained zeins from agarose IEF were analyzed by SDS-PAGE (28). In a second, IEF bands from one or more lanes were extracted with 55 μL PMA, centrifuged, and the supernatant assayed by HPLC. Thus IEF bands can be related to peaks coming off at certain times by HPLC. The extraction has proven to be rather inefficient, however, so that only some zein bands can be analyzed this way. In a third type of serial analysis, peaks were collected after preparative HPLC, freeze-dried, and redissolved either directly in SDS sample buffer or in PMA, with a fraction being put into SDS sample buffer. These were then reexamined by analytical HPLC, IEF, or SDS-PAGE to cross-identify individual zeins. Steps, such as concentration, which may cause differential losses of some zeins were avoided whenever possible.

RESULTS AND DISCUSSION

Zein Nomenclature

The six zein protein classes are often identified by the apparent molecular masses determined by SDS-PAGE. However, the use of different gel systems and different standard proteins leads to widely differing numerical assignments. These are shown in Figure 1, along with molecular masses calculated from the amino acids sequences derived from cDNA sequences. Small sample sizes show good separation of the AB zeins, but the minor zeins are then difficult to see in some lanes, especially E zein. There is no single numerical designation which is not either wrong, applied to two or more different classes of zeins, or both. I have been characterizing the individual storage proteins found in maize endosperm. I need a simple key to give each protein a unique name while avoiding the confusion caused when the differing apparent sizes determined by SDS-PAGE R_m are used as names. A satisfactory system should be expandable as new knowledge is gained. The two major zeins were called A and B, and the minor zeins C, D, E, and F in order as their properties became better known. Esen (6, 7) separated these proteins into discrete groups by careful manipulation of extractability and solubility properties, but the identity and purity of each fraction was shown by SDS-PAGE. The AB zeins are also known as the α fraction, C zein the β fraction, D zein the δ fraction, and E zein the γ fraction. The Greek names are based on solubility (6) or genetic (23) properties, with what I term F zein being assigned either to the M_r 18,000 β (7) or the M_r 16,000 γ (23) fraction, causing some confusion. I wish to concisely identify what may be called large (A) and small (B) major zeins and also to separately identify the two γ (23) (E and F) zeins. Thus the Greek and Roman letter classes divide the zein classes differently. Roman letters are more convenient to use than Greek letters, which often are not available on typewriters or word processors. The Roman letter system is neutral as to true molecular mass, and, other than the assumption that

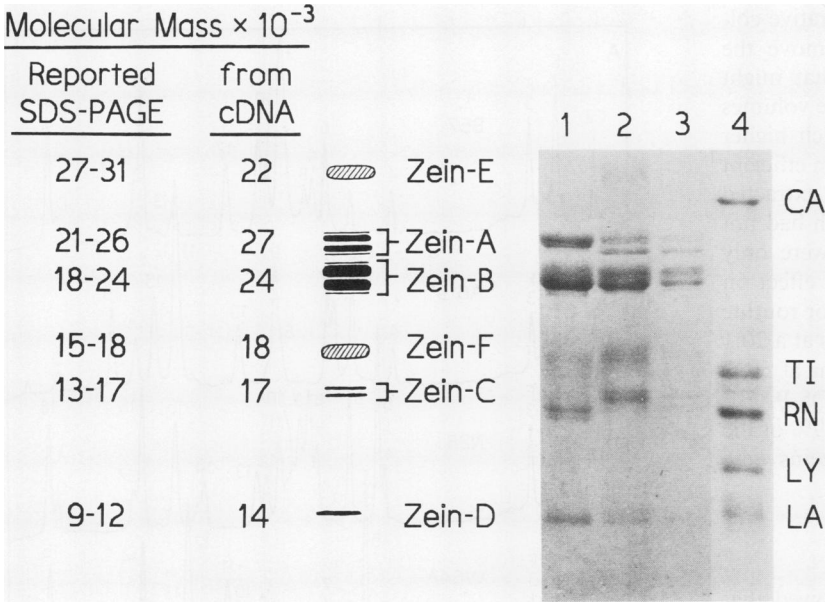

Figure 1. Idealized SDS-PAGE pattern for naming of zein classes A to F, along with true molecular masses determined from cDNA sequences and the range of apparent molecular masses reported in the literature, as determined by SDS-PAGE. On the right is a portion of an SDS-PAGE pattern (28) showing some of the separation seen in SDS-PAGE, especially for zeins C1 and C2 (lanes 2 and 1, respectively). Lane 1, M14su1; lane 2, W64Asu1; lane 3, W64A+. Standards: CA, carbonic anhydrase, 29 kD; TI, soybean trypsin inhibitor, 20.1 kD; RN, pancreatic ribonuclease, 13.7 kD; LY, lysozyme, 14.3 kD; and LA, α-lactalbumin, 14.2 kD.

the order of the zeins on SDS-PAGE will not change, does not depend upon properties of the proteins to distinguish them. If information "filed" under each name must be modified by new data, the name does not become out-dated or misleading. If other names, such as glutelin, alcohol-soluble glutelin, zein associated protein, or protein body protein, are used, the SDS-PAGE letter can be used to relate the protein to the key. These are only the main classes of the zeins. Below, I will expand the system to include possibly unique designations for each of the potential hundreds of zeins occurring in the AB zein classes. The system can also be expanded if and when additional members are found for the minor zein classes, such as the C1 and C2 zeins.

Table I (with Fig. 1, partly derived from ref. 28) presents my zein classification and a key relating it to previously used names and identifications. There are several explanations for the widely ranging values for apparent molecular masses (3, 28). I have found no good reference for the most commonly used apparent molecular masses, and there is only a partial consensus as to which numbers should be used for some zeins. Most reported values are low, but the values calculated from the right side of Figure 1 are not too far wrong for most zeins, for example.

Extraction

Originally, zeins were extracted with PM solvent because analyses were to be done on IEF, where high salt concentra-

Table I. *Classification and Key for Zein Names*

Main classes[a]	A Zein	B Zein	C Zein	D Zein	E Zein	F Zein
Subclasses	1–4	5–10	1, 2			
Mol mass, kD[b]	27	24	17	14	22	18
Apparent from SDS-PAGE[c]	21–26	18–24	13–17	9–12	27–31	15–21
Commonly used[d]	22	19	14, 15	10	27, 28	16
This laboratory[e]	26	23	17	12	31	21
Landry-Moureaux class[f]	Zein	Zein	G1	G1?	G2	G1?
Paulis-Wall term[g]	Zein	Zein	wi-ASG	wi-ASG?	ws-ASG	wi-ASG
Solubility[h]	α	α	β	δ	γ	β
Genetic class[i]	α	α	β	δ	γ	γ
Distinctive amino acid[b]	Leu Ala	Leu Ala	Met	Met	Pro His	Pro Cys
Tyrosine, mol%	3.3	3.7	8.8	0.8	2.0	5.5

[a] Classes identified and subclasses determined by special SDS-PAGE, with tentative class C′ now termed zein F (28). [b] Molecular masses and amino acid data taken from or calculated from data in refs. 10, 17, 18, 26, 29, and based on cDNA sequences. [c] Range of values taken from a number of references. [d] Apparent molecular masses from SDS-PAGE data often used to identify zeins. [e] Molecular masses determined from Figure 1. See also Figures 2 and 4 of ref. 28. [f] Ref. 11. [g] Ref. 16. [h] Refs. 6, 7. [i] Ref. 23.

tions were thought undesirable. Samples for preparative columns were preextracted with SPE solvent to remove the albumin-globulin proteins, plus other materials that might clog the column when high concentrations and large volumes were applied. I noted that E zein peaks were much higher after an SPE preextraction. Alcohol-ME-acetate is an efficient solvent for total zeins (16). PM (without acetate) extracted only a small amount of E zein from samples which had not been extracted with SPE, while the other zeins were only slightly less. Because 0.5% sodium acetate had no effect on zein separation by IEF in agarose, it was adopted for routine use. The residue from a PMA extraction (two times at a 20:1 ratio at room temperature) was extracted for one hr at 50°C with a 1% SDS sample buffer, then compared by SDS-PAGE with PMA total zein extracts (not shown). About 99% of the total AB zeins and over 90% of the C, E, and F zeins were extracted by PMA.

HPLC

Early studies with different HPLC gradients showed that different patterns were formed by each inbred, with few peaks in common. Yet the same inbreds showed more common bands by IEF. Therefore, serial analysis was used to relate each HPLC peak to zeins previously identified by SDS-PAGE and IEF. Results of these analyses on four inbreds are shown in Figure 2A and Table II. Zeins for each inbred are numbered in order of elution, to the nearest 0.1 min. The numbers assigned to a zein that is in two or more inbreds may differ for each inbred (*i.e.* the last zein in W64A, N28, and B57). Each HPLC peak was cross-identified to other analyses, using the nomenclature system described earlier (28). For example, the W64A peak at 30.0 min (R4/B7/I17.5/ch4) was a zein found in the fourth HPLC peak which occurs in main class B and subclass 7 by SDS-PAGE, position 17.5 by IEF, and its gene is located on chromosome 4 (30). This nomenclature system extends a system used elsewhere (8). The letters A to F identify zein classes separated by SDS-PAGE, and are usually given first (28), unless another element is the main subject. The IEF band number is preceded by "I" and the RP-HPLC peak number by "R," if needed to prevent confusion. Underlined peaks are those that were the same by at least two criteria. Many differences were revealed by IEF, but sometimes only SDS-PAGE (not shown) distinguished two zeins, as at 37.5 min.

Serial Analysis

The first three protein HPLC peaks were identified as wi-ASG, ws-ASG, and wi-ASG (14), but are here termed zeins C, E (formerly reduced-soluble protein), and F (C′ in ref. 28), respectively. The first peak appeared earlier from inbreds W64A and N28 than from A619 and B57 (Fig. 2). The inbreds with the early HPLC peak (termed C1) show the lower mobility C-zein on SDS-PAGE (28), while inbreds with the C2 (later) peak show the higher mobility band. These three zeins were isolated by a preparative HPLC column. SDS-PAGE of peaks 1, 2, and 3 from B57 (Fig. 3A) and A619 (Fig. 3B) confirms their identification as zeins C, E, and F, respectively. There may be some association of zeins C and F. The isolated

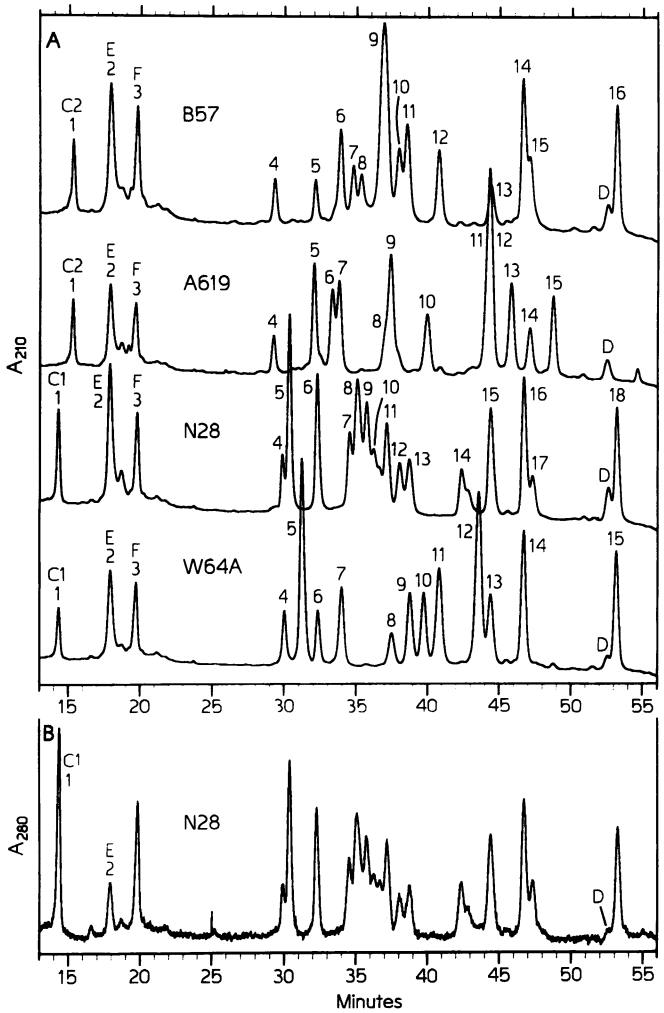

Figure 2. HPLC of zeins of inbreds W64A, N28, A619, and B57, with a segmented gradient starting at 38.3% ACN. Zeins in each peak are further described in Table II. A, A_{210}, which shows D and E zeins. B, A_{280}, detected simultaneously with the N28 sample in A, to show low absorption by D and E zeins.

C zeins were the C2 type, and can be compared with C1 from W64A (Fig. 3A, lanes 1 and 2). The AB zeins dominated the SDS-PAGE pattern of total zeins, while the minor zeins are relatively higher in a zein-2 extract. The C1-C2 differences were noted in 21 inbreds by SDS-PAGE, and the expected C1 or C2 peaks were found by HPLC for 11 of these inbreds (my unpublished data).

Relatively large amounts of AB zeins were separated by a preparative HPLC column (Fig. 4A), but the number of peaks was less than seen on analytical columns. Individual peaks were collected, freeze-dried, and dissolved in PMA for analysis by analytical HPLC or by IEF. Figure 4B matched three preparative peaks to total zein peaks separated on an analytical column. When peaks R6 and R11 (Fig. 4A) were run on IEF, both were identified as IEF band 49 (Fig. 4C). Peak R10 matched IEF band 20. However, the large peak at 27 min revealed four IEF bands from the ascending side and five bands from the descending side.

Table II. *Identification[a] of Zeins in HPLC Peaks shown in Figure 2*

Min	Inbreds			
	W64A	N28	A619	B57
14.3	R1/C1/I42[b]/–	R1/C1/I42/–		
15.3			R1/C2/I42/–	R1/C2/I42/–
17.9	R2/E/–/–	R2/E/–/–	R2/E/–/–	R2/E/–/–
19.7	R3/F/I37[b]/–	R3/F/–/–	R3/F/–/–	R3/F/–/–
29.3			R4/A2/I44.5/–	R4/A2/I44.5/–
29.8		R4/A2/I44.5/–		
30.0	R4/B7/I17.5/ch4			
30.3		R5/B6/I32/ch4		
31.2	R5/B9/I44/–			
32.0			R5/–/I44/–	R5/–/I44/–
32.3	R6/–/I45/–	R6/B8/I54/–		
33.3			R6/A3/I14+18/ch4	
33.8			R7/B9/I49/ch4	R6/B9/I49/ch4
34.0	R7/B8/I32/ch4			
34.5		R7/B8/I36/ch4		
34.7				R7/–/–/–
35.0		R8/B7/I36/ch4		
35.3				R8/–/–/–
35.7		R9/B9/I49/ch4		
36.2		R10/A1/I43.5/–		
36.9			R8/A1/I43.5/–	R9/A2/I28/ch4
37.2		R11/–/–/–		
37.5	R8/B10/I35/–		R9/B9/I35/ch4	
38.0		R12/A1/I20/–		R10/A1/I20/–
38.5				R11/B9/I49/ch4
38.7	R9/–/–/–	R13/B7/I36/ch4		
39.7	R10/A2/I60/ch4			
39.9			R10/A1/I20/–	
40.8	R11/B6/I36/ch4			R12/A3/I20/–
42.4		R14/–/–/–		
43.5	R12/A3/I33.5/ch4			
44.0			R11/A3/I20/–	
44.4	R13/A1/I20/–	R15/A3/I33.5/ch4	R12/B9/I10/ch7	R13/A1/I20/–
45.8			R13/B9/I38/–	
46.7	R14/B9/I38/ch7	R16/B9/I38/ch7		R14/B9/I38/ch7
47.2		R17/A1/I37/–	R11/A1/I37/–	R15/A1/I37/–
48.7			R15/B9/I22/ch7	
52.6	[c]–/D/I55/ch9	–/D/I55/ch9	–/D/I55/ch9	–/D/I55/ch9
53.2	R15/B9/I38/ch7	R18/B9/I38/ch7		R16/B9/I38/ch7

[a] The identification sequence is: R, RP-HPLC sequential number/A to F, SDS-PAGE major zein class followed by numerical subclass (Fig. 1)/I, IEF band number (28)/ch, chromosome number for structural gene (1, 30). Underlining marks zeins for which all elements, including time and at least one other, match. [b] The C and F zein positions on IEF differ, relative to AB zeins, for different runs and for different lots of Ampholine. [c] No R number is given to D zein because its position, relative to AB zeins, varies with minor changes in gradient conditions.

Serial analyses were done in the reverse direction by extracting the zeins from stained and dried agarose IEF bands with PMA. Coomassie blue R produced some early peaks (not shown), but did not interfere with the zein peaks. The alcoholic extract was mixed with SDS buffer for analysis by SDS-PAGE. It was thus possible to cross-identify many analytical HPLC peaks. Although some IEF bands contained zein that eluted in a single HPLC peak, Figure 4D shows an IEF band from the inbred N28 that produced four peaks by HPLC (peaks R7, 8, 9, 11, Table II and Fig. 2). These same four peaks were also found in the IEF band 36 of unrelated inbreds Va35 and Pa91 (not shown). Band 36 from W64A and from the related inbred WF9 formed a single HPLC peak (R11 in Table II and Fig. 2) at a different time. Furthermore, inbreds B14A, B84, and R801 (derived from the same synthetic line) had an IEF band 36 which gave two additional HPLC peaks (not shown). Thus zeins which moved to one position by IEF and to two positions by SDS-PAGE (28) were found in seven HPLC peaks. Preparative HPLC peaks 4 and 5 gave single bands by IEF (Fig. 4C).

The differences among the AB zeins of W64A, N28, A619, and B57 are summarized in Tables III and IV. By IEF, I detected from 7 to 13 different zein bands in each inbred; because some appeared identical, however, the total for all

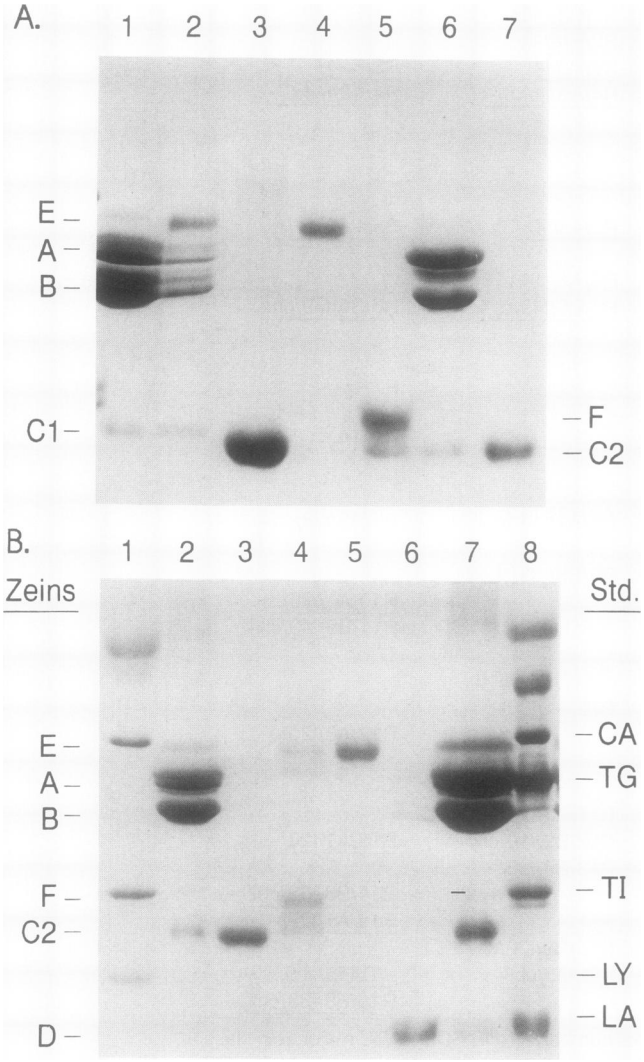

Figure 3. SDS-PAGE of C, D, E, and F zein fractions from preparative HPLC with a segmented gradient starting at 34.37%. Gels were stained with the Coomassie blue G reagent (12). A, Inbred B57: lane 1, whole zein from W64A, with C1 zein: lane 2, zein-2 (extraction with PM after extraction with isopropanol only) from W64A; lane 3, C2 zein (from 1.7 mg B57 endosperm); lane 4, E zein (0.3 mg); lane 5, F zein (1.7 mg); lane 6, whole zein from B57; lane 7, C2 zein (0.7 mg). B, Inbred A619: lane 1, Bio-Rad standard proteins; lane 2, A619, whole zein; lane 3, C2 zein (0.7 mg); lane 4, F zein (1.1 mg); lane 5, E zein (0.7 mg); lane 6, D zein (2.5 mg); lane 7, A619 whole zein, with F zein marked because reproduction may not be visible; lane 8, Sigma protein standards. See Figure 1 for identification of standard proteins, plus TG, trypsinogen.

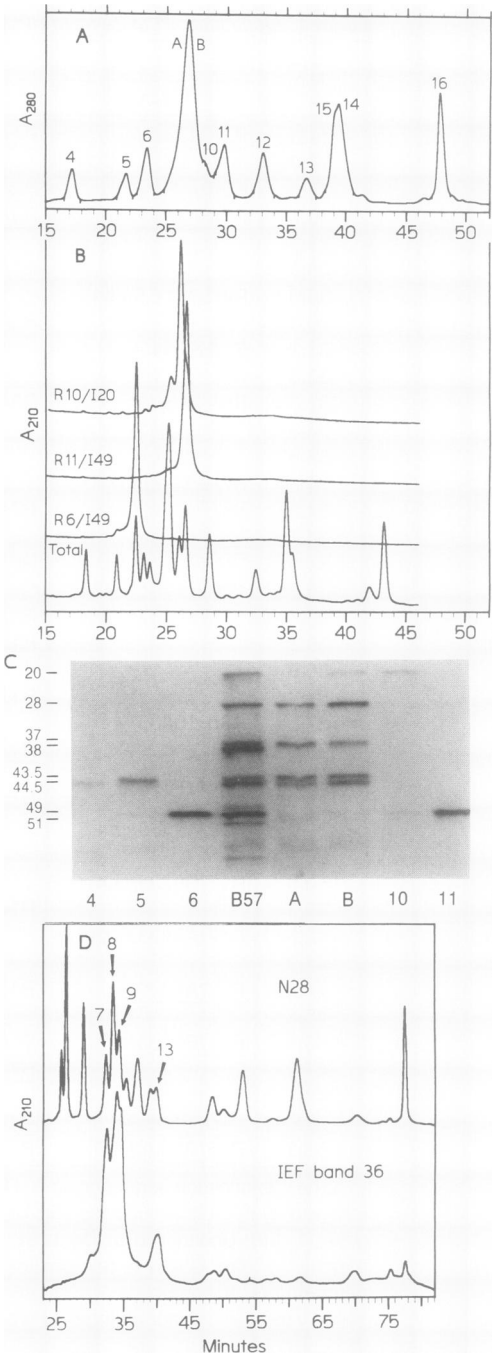

Figure 4. Serial analysis of several zeins by preparative HPLC, analytical HPLC, and IEF. A, Preparative HPLC, starting at 40% ACN, of supernatent solution from 30 mg SPE-extracted B57 endosperm in 150 μL PM solvent. The numbers match those of the analytical column (Fig. 2), except that the largest peak is subdivided into two fractions A and B. Fraction collection limits marked. B, Serial analysis of three preparative HPLC fractions by analytical HPLC, compared to the pattern of total B57 AB zeins. C, Serial analysis of several B57 preparative HPLC fractions by IEF. The sample numbers correspond to peaks in Figures 2A and 4A. The vertical scale gives mm from the cathode of zein bands on a "typical" gel (28). D, Serial analysis by extraction of zein from a single IEF band (band I36 of inbred N28) and subsequent analysis by analytical HPLC, with comparison to total N28 zein (an experimental gradient).

Table III. *Number of Identifiable AB Zeins in Four Inbreds*

Inbred	Assay		
	IEF	HPLC	Serial[a]
W64A	10	12	12
N28	11	15	15
A619	13	12	13
B57	7	13	13
Total	23	34	40

[a] Serial analysis: successive assays by IEF, HPLC, and SDS-PAGE.

four inbreds was only 23 (27). HPLC alone separated a total of 34 zeins. Serial analysis by IEF, HPLC, and SDS-PAGE did not increase the number of identifiable zeins in any inbred, but the total of different zeins among the four inbreds was increased to 40. The numbers of zeins which match between the inbreds is contrasted with the total number of zeins identified for each pair in Table IV. HPLC separated more total zeins for each pair than does IEF, but the number of matching zeins was higher for only one pair. More zeins were identified when all three assays are used, while the number of matching zeins was decreased. Two pairs, N28-B57 and B57-A619, shared four zeins, while one pair, W64A-A619, had no zeins in common. No single zein occurred in all four inbreds, three were in three inbreds, and six were found in two inbreds. At the other extreme, Oh43 and its daughter inbred A619 shared all 13 identified zeins.

D Zein Isolation

The D zein HPLC peak was located using the inbred BSSS-53, which has a high level of this zein (1). The I55 band produced on agarose IEF was known to contain both D zein and a B5 zein (my unpublished data). The agarose strip with I55 was extracted with PMA and the extract was assayed by analytical HPLC. Two peaks absorbing at 210 nm were found, at 51 and 52.6 min, but the second peak was almost undetectable above the baseline at 280 nm. Only Trp and Tyr contribute to A_{280}, but the zeins have no Trp, except for F zein (18). Of all zeins, D zein has the least Tyr (Table I), and is thus expected to have the lowest A_{280}/A_{210} ratio. The I55 second peak also partially overlapped the final major peak from BSSS53. Fortunately, inbred A619 does not contain a major HPLC peak at this time, so the low A_{280} zein peak occurred alone. This peak was collected from a preparative column, freeze-dried, redissolved in SDS sample buffer, and run on SDS-PAGE (Lane 6, Fig. 3B). It was identified as D zein. Note that D zein could be named a 10-kD zein if compared to lysozyme, but it would be named a 14-kD zein if compared to α-lactalbumin. Reexamination of several HPLC analyses showed that D zein, identified by low A_{280}, was present in most inbreds, but was sometimes obscured by the major I38 zein found in many inbreds. A preliminary test suggests that D zein may be separated from other zeins by changing the slope of the gradient. D zein was not assigned an R number because its position relative to AB zeins fluctuates. D zein was mapped to chromosome 7 (8, 30), but more recent work mapped D zein to chromosome 9 (1).

Table IV. *Comparison of the Number of AB Zeins Matched by Individual and Serial Analysis with the Total Number of Different Zeins in Pairs of Inbreds*

Inbred Pairs	Matching Zeins/Total Zeins		
	IEF	HPLC	Serial[a]
W64A-N28	5/16	5/22	2/25
W64A-A619	4/19	1/23	0/24
W64A-B57	2/15	4/21	3/21
N28-A619	5/19	1/26	1/30
N28-B57	6/12	5/23	4/24
A619-B57	5/15	3/22	4/22

[a] Serial analysis: successive assays by IEF, HPLC, and SDS-PAGE.

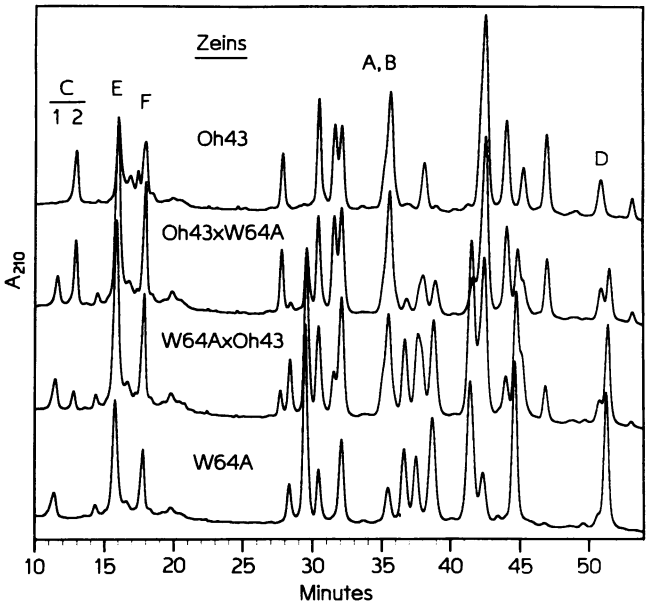

Figure 5. Analytical HPLC of zeins from two inbreds and their reciprocal F_1 hybrids. The gradient started at 34.37% ACN.

Identification of Zeins by the A_{210}/A_{280} Ratio

A comparison of the A_{280} elution pattern for the inbred N28 with the A_{210} pattern (Fig. 2, A and B) correlated with the % Tyr. The C zein peak was increased relative to the other zeins at A_{280}, the E zein peak was greatly reduced, and the F zein was slightly increased. The AB zeins were alike at both wavelengths.

HPLC Patterns of Hybrids

Figure 5 compares inbreds Oh43 and W64A and their reciprocal hybrids. All parental zeins were present at the expected dosage levels in the hybrids. Zeins C1 and C2 behaved as if their genes were allelic at the same locus. Smith and Smith (22) reported similar findings for the AB zeins only, and discussed the usefulness of HPLC profiles for checking pedigrees.

Quantitative Analysis of Zein by RP-HPLC

A single simple extractant, PMA, efficiently extracts all classes of zeins. The extract can be directly assayed without further treatments which might differentially affect zeins. UV absorption at 210 nm is due largely to peptide bonds, and is only slightly affected by the amino acid composition of individual proteins (2). Calculations suggest variability of 10% or less among the zein classes. Thus, HPLC directly yields data which are proportional to the actual contents of the different zeins. Work to be done includes calibration of the peak area at 210 nm determined by our instrument to a given amount of protein (2). Only relative peak areas can be determined now.

Estimates of the relative amounts of the zein classes in five inbreds are presented in Table V. The AB zein fraction ranges from 71 to 84%, with the remainder being made up of varying proportions of the minor zeins. There is a fourfold range of the high methionine C and D zeins and a twofold range for the E and F zeins. Mo17 differs from the others in having a very high level of E zein and low levels of C, D, and F zein. BSSS-53 has high levels of all four minor zeins, including the expected high level of D zein (1). The reproducibility of the method is shown by the small range of values obtained for M14 samples stored for 6 and 8 years and freshly harvested. RP-HPLC should be a useful method for breeding programs attempting to raised the level of methionine in corn storage proteins. Appreciable differences do occur in the levels of the different zein classes in selected nonmutant inbreds.

Genetic Relationships

AB zeins are encoded by many genes grouped into several multigene families (9, 19, 23). Although the number of genes is estimated to be over 100, some may be pseudogenes. HPLC coupled with IEF and SDS-PAGE detected 40 different AB zeins in only the four inbreds. Furthermore, not a single zein occurred in all four inbreds, and no more than 4 zeins were shared by any two inbreds, out of totals of 21 to 30 zeins (Table IV). Examination of other inbreds suggests that many more different AB zein genes are indeed functional in various inbreds. The AB zeins appeared to be inherited normally, so that hybrids can be expected to contain all of the zeins of their parents, but no new zeins. Some closely related inbreds, such as Oh43 and A619, derived from Oh43, had identical patterns for all zeins. HPLC can serve as a powerful tool for determining whether corn lines are related, though it must be recognized that zeins are good markers only for chromosomes 4 and 7.

AB zein genes, mapped using agarose IEF, occur in two linkage groups on chromosome 4 and one on chromosome 7 (30). It was first assumed that each IEF band represented the product of a single gene that was the same in all inbreds containing the band, so data from several inbreds were combined. These data were reexamined when HPLC revealed that bands I32 and I33.5 from W64A (R7 and R12) and N28 (R5 and R15) were different. I36, tightly linked to I32 (30), separated into four peaks from N28 (Fig. 4D), while W64A had one peak which differed from all four. However, both inbreds showed 25% crossovers between I32 and I33.5. With other data, it appeared that these genes would be found in the same positions on the chromosome in these two inbreds. However, bands I32 and I49 gave differing crossover data when certain inbreds were involved (30). Thus, genetic mapping by IEF of zeins in a few inbreds does not give data which can be assumed to be correct for other inbreds which have zeins at the same IEF positions.

IEF band 38 is one of the most common zein bands, but HPLC separated it into two peaks late in the gradient (Table II, Fig. 2). HPLC assays, without serial analysis, of 18 inbreds found two peaks at the I38 positions in 9 inbreds, while three inbreds had a single I38 peak. All of the inbreds which lacked two I38 HPLC peaks have bands I10 plus I22, or band I21 or I21.5. These are all chromosome 7 zeins (ref. 30 and my unpublished data).

Some suggestions may be made concerning the changes in amino acid compositions which alter separations of zeins obtained with the three systems. As storage proteins, zeins are less subject to selection after mutation than are enzyme proteins. However, all of the zein classes have similar signal peptides, which are required for proper targeting of the protein bodies where zeins are deposited (10). AB zeins also have repeat units of about 20 amino acids. The major size difference detected by SDS-PAGE for A and B zeins is due to the presence of one more repeat unit in the A zeins. Zein genes may be duplicated and then one of the resulting genes may undergo a base substitution (21). If the modifications cause a change in a charged amino acid, the resulting zein would migrate to a different position upon IEF. If the change involves only neutral amino acids, a change in hydrophobicity might occur. For example, a single base change could change

Table V. *HPLC Zein Classes as Percentage[a] of Total Zeins*

Inbred	Zein				
	AB	C	D	E	F
			%		
W64A (3)[b]	84.5 ± 1.6	C1 2.6 ± 0.5	1.0 ± 0.1	6.8 ± 1.3	5.1 ± 0.6
Oh43 (2)	78.9 ± 0.1	C2 4.0 ± 0.2	3.4	8.6 ± 0.1	5.1
Mo17 (2)	78.6 ± 2.4	C1 1.0 ± 0.5	0.9	14.8 ± 0.1	3.6 ± 0.6
M14 (3)[c]	76.8 ± 1.2	C2 4.0 ± 0.6	3.1 ± 0.3	10.1 ± 0.7	6.0 ± 0.2
BSSS-53 (2)	71.0 ± 1.2	C1 5.0 ± 0.3	4.6 ± 0.3	11.0 ± 0.4	8.4 ± 0.8

[a] Averages ± the range of values. [b] Number of samples indicated in parentheses. [c] Samples harvested in 1982, 1984, and 1990.

Gln into Leu. The more hydrophobic Leu binds more SDS, and thus causes more rapid migration in SDS-PAGE (5). This may cause the minor differences in AB zein mobilities sometimes reported (7, 28). Increased hydrophobicity increases the binding of zein to an RP-HPLC column, leading to another separation of a new zein from the original form. The chromosome 7 zeins so far characterized by serial analysis are eluted late in the profile, indicating above average hydrophobicity. The zeins which form bands I32 and I36, which are tightly linked in one linkage group on chromosome 4, might form a single genetic subfamily as a result of repeated duplication and modification of one zein gene, for I36. I32 from W64A and N28 might result from the introduction of a basic amino acid (changing the IEF position) followed by one mutation changing hydrophobicity (thus changing the HPLC elution position). The I36 gene in N28 might have undergone repeated duplication and mutation to end with four proteins differing in hydrophobicity. This assumes that duplicated genes remain tightly linked in the same position in both inbreds.

Many single and double endosperm mutants cause decreases in AB zeins and increases in C, E, or F zeins which are detected by HPLC (15). Wallace, et al. (25) extracted the minor zeins with a new SDS-based extraction system. E zein was relatively high in modified opaque-2 maize, as determined by an immunological assay. However, HPLC also quantitatively detected similar differences after a simple ethanol-ME-acetate extraction of modified opaque-2 lines (JW Paulis and JA Bietz, unpublished results).

Comparison of Zein Assays

Four assays can be used in detailed studies of zeins. SDS-PAGE uses equipment which is widely available and separates the six classes of zeins in one operation. Separations are based on size, but other factors also influence mobility. The naming of zein classes according to the apparent molecular masses determined by SDS-PAGE has led to confusion among the different numbers being used. Hydrophobicity (5) and protein shape influenced by high proline contents may contribute most to erroneous molecular mass data for zeins. Some differences among inbred patterns may be detected by SDS-PAGE, but these are difficult to reproduce (28). SDS-PAGE is not quantitative, because most commonly applied staining procedures do not completely stain proteins (28) and the color yield of most zeins is not known. The AB zeins absorb less than one-fourth as much Coomassie blue R and G than do most proteins (29). Nothing is known about dye-binding characteristics of the other zeins.

IEF is widely available, but it separates only the AB and D zeins. C and F zeins occur occasionally on agarose IEF gels, but are subject to variable expression (28). This may be related to the difficulty of keeping them in solution (6, 7). E zein may or may not be seen. Classical linkage studies of AB zeins can be done with IEF, but the data cannot be applied with certainty to any inbreds other than those actually tested. Zeins from over 50 samples can be assayed simultaneously by matching against standard inbreds (27). Actual separation distances can vary with different ampholytes and different batches of the same commercial ampholyte. Two-dimensional (IEF followed by SDS-PAGE) separations of zeins from different lines are difficult to compare in detail to identify zeins.

Immunological assays have been proposed for quantitative assay of zeins (4, 25). The required antibodies are not widely available, and results are difficult to express in absolute terms. Antibodies to C, D, E, and F zeins may be satisfactory because there seems to be little genetic variability among genotypes. The AB zeins exist in many forms having 50 to more than 95% sequence similarity, and are grouped into as many as six genetic subfamilies. Thus it may be difficult or impossible to have an immunological assay that quantitatively assays all AB zeins in any maize line.

The major drawback to routine HPLC assay of zeins is the expensive equipment required. Exact reproducibility requires careful mixing of buffers, which may change with age. Some gradient changes do not seem to affect the resulting AB zein profiles. However, other gradients improved separation of some zeins, while separation of other zeins decreased. No comprehensive studies have yet been done on the reproducibility of HPLC profiles using different types of columns from different suppliers. This lack of exact reproducibility makes precise identification of individual zeins difficult and requires frequent running of a standard inbred for comparison. W64A is a good HPLC standard in that its AB zeins tend to be spaced over the entire gradient. Also, most W64A peaks contain only one zein. Absorbance at 210 nm is proportional to the amount of protein (2), while relative absorbance at 280 nm provides an identification for a zein peak in addition to elution position. This should prove useful in a search for lines with high C and D zeins, and thus high methionine, for example.

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