Improved Two-Dimensional Electrophoretic Separation of Zein Proteins: Application to Study of Zein Inheritance in Corn Genotypes[1]

J. S. WALL, D. A. FEY, and J. W. PAULIS, Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture,[2] Peoria, IL 61604; and J. LANDRY, Laboratoire de Technologie Alimentoire INRA, Massey, France

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ABSTRACT

The heterogeneity and genetic variability of polypeptides constituting the protein zein of corn (*Zea mays* L.) were demonstrated by two-dimensional polyacrylamide gel electrophoresis consisting of isoelectric focusing in the first direction and migration in aluminum lactate pH 3.5 buffer in the second. Zein was extracted from ground corn endosperm with 70% ethanol; the protein was reduced and its sulfhydryls alkylated. Isoelectric focusing was performed in thin gel slabs containing Ampholines (pH 6–8) in 6 *M* urea at 4°C with constant power. Strips containing resolved zeins were cut from gel slabs, equilibrated with second-dimension buffer, and then inserted into slots in pre-electrophoresed aluminum lactate (pH 3.5) 8*M* urea gel slabs. After electrophoresis at 4°C and constant power, the 25–40 different zein polypeptides were made visible by silver staining. Zeins from extracts of different inbreds have some different components. Electrophoretic patterns of zeins from hybrids contained polypeptides from both parents, but the female's contribution was quantitatively greater. Inbreds that were derived from crosses usually contained some zeins from both parents. Electrophoretic patterns can characterize zeins in corn inbreds and can aid in relating corn hybrids and inbreds to parental lines.

Zein, the 70% ethanol-soluble, water-insoluble protein of corn (*Zea mays* L.) endosperm, constitutes more than 35% of the grain protein (Wall and Paulis 1978). Using starch gel electrophoresis in aluminum lactate buffer containing 8*M* urea, Turner et al (1965) first demonstrated that zein was heterogeneous. Alexandrescu et al (1976) and Paulis and Wall (1977) used polyacrylamide gel electrophoresis (PAGE) to resolve zeins and observed differences in the composition of zeins from different inbreds and varieties. Further complexity of zein was demonstrated by Righetti et al (1977) by isoelectric focusing (IEF) in gels that resolved 10–15 components. Nucca et al (1978) demonstrated that the IEF patterns of zeins were characteristic of many inbreds and could be used to establish genetic relationships.

Zein polypeptides do not vary greatly in molecular size. Misra and Mertz (1976) and Burr and Burr (1976) detected only two fractions differing in molecular weight during electrophoresis in 10–14% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol (ME); the lower-molecular-weight component was approximately 19,000–20,000, and the higher was 21,000–22,000. In addition to these major bands, Lee et al (1976) observed minor components with molecular weights 15,000, 14,000, and 11,000 in alcoholic extracts containing ME or in extracts prepared at elevated temperature. To correlate the different molecular weight species with the numerous proteins separated by charge, Hagen and Rubenstein (1980) and Wilson et al (1981) resorted to two-dimensional (2-D) electrophoresis—IEF in the first direction and SDS-PAGE in the second. This system, based on the method of O'Farrell (1975), yielded 15–25 components of reduced zein and established that both major-molecular-weight species of zein were heterogeneous in charge. This method was still not optimal for genetic studies because some zeins coded for by different genes apparently migrated to the same electrophoretic site.

The use of 2-D electrophoresis, IEF in the first direction and pH 3.5 aluminum lactate buffer PAGE in the second direction, greatly increased the number of resolved zein components. This electrophoretic system is similar to one used by Wrigley and Shepherd (1973) on starch gel for wheat gliadin analyses. Silver staining (Merrill et al 1981) to detect the separated proteins increased the sensitivity of the procedure and yielded color differences in the separated spots that facilitated identification of specific zeins. This paper describes a simplified procedure for attaining 2-D electrophoretic separation of zeins by IEF followed by PAGE in acidic buffer. Examples are given of its use to differentiate corn inbreds through analysis of the extracted zeins. The compositions of zeins from hybrids and derived inbreds are compared to those of the parental lines. Also, the taxonomic relationships between some major U.S. corn inbreds and breeding groups based on early elite varieties are explored based on the 2-D electrophoretic patterns of their zeins.

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[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.

MATERIALS AND METHODS

Preparation of Zeins

Samples of seeds of corn inbreds and hybrids were obtained from R. I. Brawn, Funk Hybrid Seed Co., Bloomington, IL, and from D. E. Glover, Department of Agronomy, Purdue University, W. Lafayette, IN. Six kernels from each line of corn were soaked for 1 hr in water; then endosperms were manually dissected, air-dried, and ground in a micro-Wiley mill to −60 mesh. The endosperm meal was mixed with 10 ml of 70% ethanol in a 16-ml sealed screw-cap tube and extracted for 2 hr at room temperature on a reciprocating shaker. The mixture was centrifuged with a benchtop International Centrifuge (model CL) for 10 min. The decanted supernatant was dialyzed for 4 hr against water and then overnight against fresh water at 4°C. The entire content of the dialysis bag was lyophilized to dryness. The extraction can be performed on a single kernel when desired (Paulis and Wall 1979).

To ensure that the polypeptides were in a monomeric form before IEF or PAGE, they were reduced, and sulfhydryl groups were alkylated with acrylonitrile (Paulis and Wall 1977). The 0.13-ml reaction solution contained 2 mg of zein for standard IEF or PAGE analysis and 6-mg sample for 2-D electrophoresis.

Electrophoresis

The procedure of Righetti et al (1977) was used for IEF both for one-dimensional analysis and for preparation of strips for use as first-dimensional separations in the 2-D system. All separations were performed on an LKB Multiphor (model 2117) electrophoresis apparatus using 27 × 11.5 × 0.2-cm slab gels. A sheet of plastic support foil was inserted between one glass plate and the gasket of the gel-forming cell to facilitate gel handling. The IEF gels contained 5% acrylamide, 6*M* urea, and 2% LKB Ampholines (pH 6–8). IEF was run in the direction of the narrow width of the gel. The gel (with plastic backing down) was placed on a plastic grid sheet coated with Amosol on the cooling plate. The positive electrode wick was soaked in saturated aspartic acid solution and the negative electrode with 0.1 *M* lysine. Samples for

analytical and 2-D runs were absorbed on 1.0 × 0.5 cm cambrele sponge wicks placed 1 cm from the positive electrode. IEF was conducted at 4°C at 13 W constant power for 4 hr. After the run, the pH of the gel was measured at 1-cm intervals between electrodes with a surface contact electrode while the gel was on the cooling surface. Gel strips (9 cm) for the second dimension were sectioned lengthwise from the center of the IEF sample pathways with the aid of a double blade with edges spaced 2 m*M* apart. Two strips were then separated, each on its own piece of plastic, and used immediately in the second dimension. Other portions of the gel were left on the remaining plastic sheet, sealed in Saran wrap, and stored for later use by freezing at −80°C.

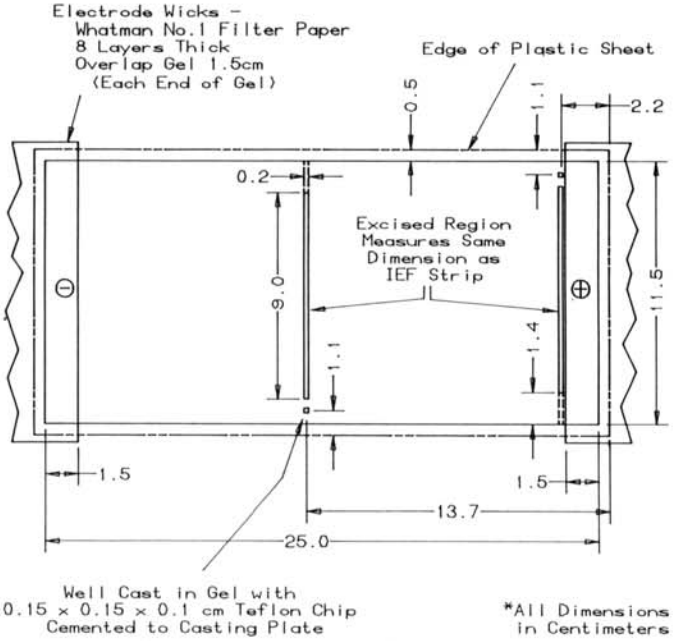

Fig. 1. Gel dimensions for second-dimension aluminum lactate electrophoresis and position of first-dimension gel strip insertions.

For second-dimension PAGE (aluminum lactate buffer, pH 3.5), the slab-forming cell was the same except that one glass plate was slightly modified to carry two small Teflon chips (0.15 × 0.15 × 0.10 cm) to cast wells in the gel for reference samples (Fig. 1). The aluminum lactate gel solution consisted of 28.83 g of urea, 2.53 g of acrylamide, 0.154 g of N,N′-methylenebisacrylamide, 0.0295 g of aluminum lactate, 0.25 ml of lactic acid, and 225 μl of Temed (N,N,N′,N′-tetramethyl-ethylenediamine) made to 60 ml with deionized water. This solution was degassed, 1.3 ml of 1% ammonium persulfate was added, and the solution was introduced immediately into the slab-forming cell. Polymerization was allowed to proceed for 2 hr.

The troughs at the long ends of the Multiphor unit were filled with aluminum lactate buffer consisting of 0.884 g of aluminum lactate, 3.6 ml of lactic acid, and 432.4 g of urea diluted to 1,800 ml with deionized water. The gel was centered on a grid sheet coated with Amosol and placed on the cooling plate with the negative end nearest the cooling outlet. Two electrode wicks, each eight layers of Whatman No. 1 filter paper, were saturated with reservoir buffer and placed to overlap 1.5 cm of each end of the gel. Heavy Plexiglas strips were set on the ends to keep the wicks on the gel. Tracking dye (1% Safranin O solution) was introduced at the positive end of the gel. A sealed anticondensation lid was set over the gel and the main lid over the apparatus. The gel was pre-electrophoresed for 6 hr at 13 W constant power at 4°C.

Following pre-electrophoresis, two slots the same dimensions as the IEF strips were cut in the gel with the aid of the same double blade, as shown in Fig. 1. Two separated IEF strips on plastic

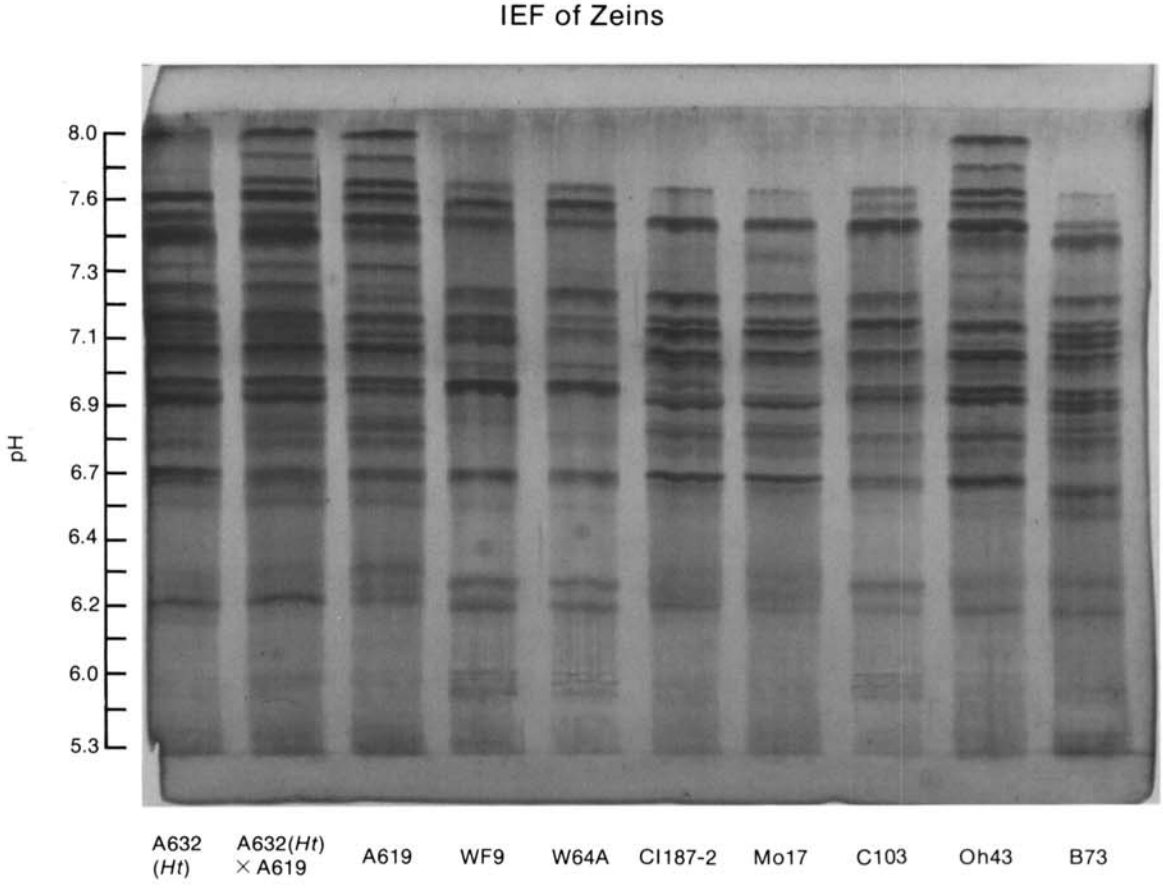

Fig. 2. Isoelectric focusing patterns of zeins from various inbred and hybrid corns. Measured pH of gel is shown on left. Cathode is at top.

support foils were immersed in aluminum lactate buffer for 1.5 min. Each strip was introduced into its slot in the second-dimension gel with correct orientation. Air spaces were pressed out and excess liquid carefully removed with filter paper. Reference sample (2 μl) was introduced into a well adjacent to IEF strip insertion, and tracking dye was applied. After replacing anticondensation lid and cover, electrophoresis was performed at 4°C and 7.8 W constant power for 16 hr.

Silver staining of the gels according to Merrill et al (1981) with Bio-Rad silver stain reagents followed the protocol of the manufacturer (Bio-Rad Laboratories 1982). Each large gel thus yielded two 2-D electrophoretic patterns, which were separated for photography.

Calculation of Agreement Among 2-D Patterns

Relationships between corn genotypes were examined on the basis of zein electrophoretic patterns by a method suggested by that of Hamann (Sokal and Sneath 1963). Photographs of the 2-D patterns of zeins from each of 10 inbreds were examined. Approximate positions of each spot representing a protein were recorded on a grid on a separate transparent plastic sheet for each inbred. For the entire set of 10 patterns examined, the location of each spot, the total number of each spot, and the number of times a spot appeared at each component site was recorded on another transparent grid. For two different inbreds, the following three combinations were possible at any given protein site: both of the pairs showed a spot at a specific site (Hit or H); only one showed a

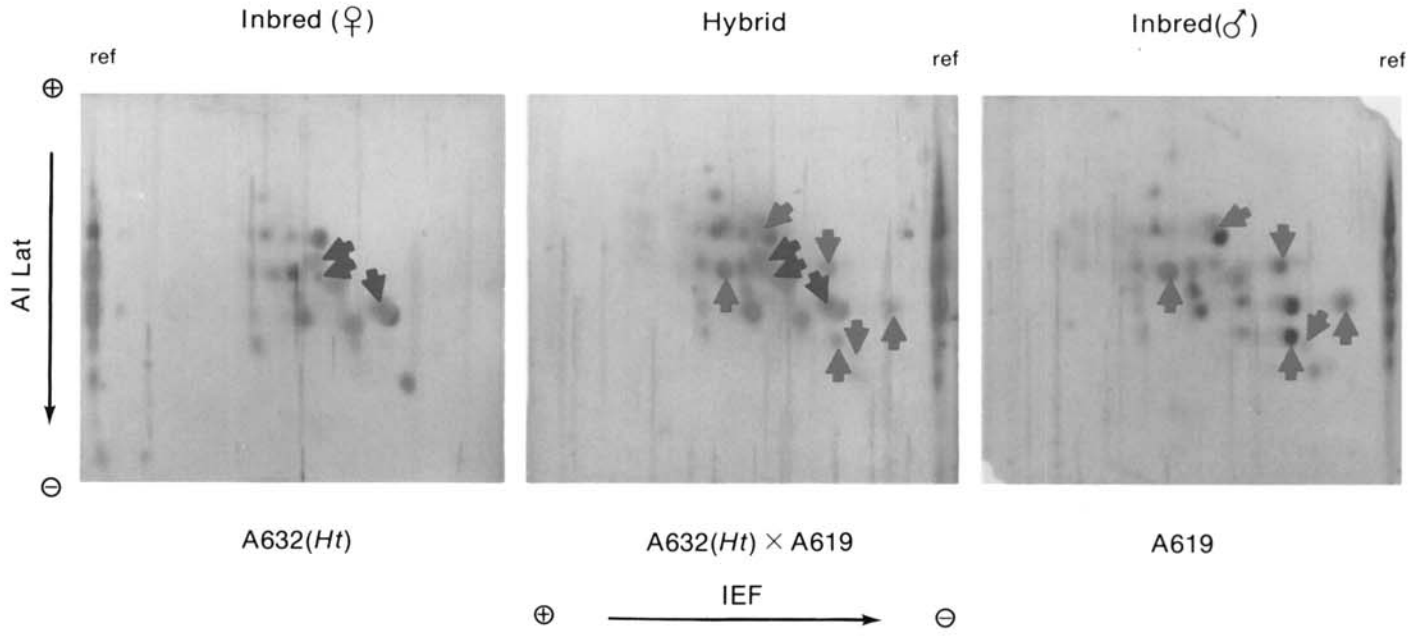

Fig. 3. Comparison of two-dimensional electrophoresis patterns of zeins from corn inbreds A632(*Ht*) and A619 and from hybrid A632(*Ht*) × A619. Ampholine pH 6–8. Electrode positions and direction of migration as shown. Blue arrows indicate proteins unique to A632; green arrows indicate proteins unique to A619.

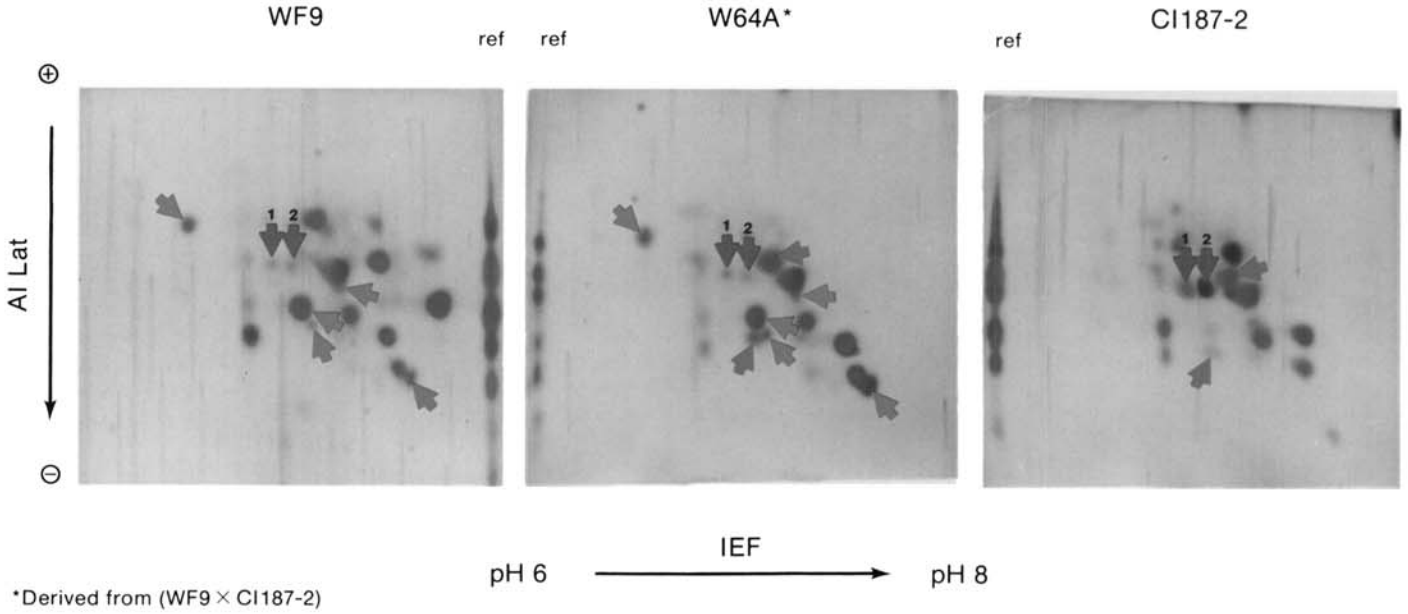

Fig. 4. Comparison of two-dimensional electrophoresis patterns of zeins from inbreds WF9, W64A, and CI187-2. W64A is derived from a cross between WF9 and CI187-2. Green arrows indicate proteins derived from WF9, and red arrows indicate proteins from CI187-2 in W64A. Blue arrows (1,2) show proteins at same coordinates in WF9 and CI187-2 that differ in size and staining.

spot at that site (Miss or M); and neither showed a spot at the site (Absence or A). Protein spots in each pair of inbreds were given a weighted value for each of these possibilities.

For any two inbreds under comparison, the number of H's indicate the degree of relationship. Fewer total spots in a locus indicates that an H has greater significance as a relationship factor. The weighted assigned value (V) for the H, designated VH, is $VH = n - C_i$, n = total number of inbred patterns examined, and C_i = number of patterns having spot at site i. Absence of an electrophoretic spot at a locus (A) in patterns of a pair of inbreds indicates a closer relationship. The more frequently the spot occurred among the 10 selections, the greater the chance that a pair of inbreds would exhibit it. Therefore, a greater weighted value (V) is given for its absence, VA, as $VA = C_i$. The occurrence of a spot present at a locus in the electrophoretic pattern of one inbred and not in that of the other inbred being compared is distinct evidence for partial dissimilarity of the inbreds' zeins. The more spots present at a locus for all 10 inbreds, the less chance for an M among two inbreds and so the VM is assigned a higher value: $VM_i = C_i$. If site 6 of our composite grid has C = 7 (a spot appeared there seven times), and we examined a total of 10 patterns (n = 10), for any pair of patterns exhibiting an H at site 6, then $VH_6 = 10 - 7 = 3$; for any pair having an M at site 6, $VM_6 = 7$; and for any pair having an A at site 6, $VA_6 = 7$.

For each possible pair of patterns in the set an Agreement Index (AI) was calculated:

$$AI = \Sigma\ VH_i + \Sigma\ VA_i - \Sigma\ VM_i$$

AI's were then transformed into more convenient positive numbers by adding a constant to all AI's.

AI's were listed in order of highest to lowest value for each pair. Pairs having similar AI's that share at least one member of the pair in common were grouped together. A dendrograph was drawn joining groups at the appropriate AI value.

RESULTS

Isoelectric Focusing

Figure 2 shows the IEF patterns of the zeins extracted from some corns examined in this study. Each of the inbred lines has a different pattern. On some of the more complex patterns, such as A619, as many as 28 different bands may be distinguished. The differences in color achieved by silver staining make visible different bands that are in close proximity. Similarities in bands with similar pI's and color are noted in inbreds that are related by parentage, such as WF9 and W64A and A619 and Oh43. Furthermore, the hybrid A632 (*Ht*) × A619 contains all bands present in both parents. These observations are consistent with those of Motto et al (1979). However, the proximity of certain bands makes some zein proteins difficult to differentiate.

2-D Electrophoretic Separation

The 2-D electrophoretic separation of zeins extracted from endosperm of inbred A632(*Ht*) is shown in Fig. 3. The first-dimension IEF strip inserted in the gel above this pattern contained a separation identical to that of the first pathway on the left of Fig. 2. IEF orientation is indicated by the arrow at the bottom of Fig. 3. The acidic-PAGE separation of the zein extract ("ref") is shown on the left; direction of movement is indicated by the arrow labeled "Al Lat." Some 13 spots are resolved in the one-dimensional separation. A632(*Ht*) zein was resolved into 25 different spots by 2-D electrophoresis with colors ranging from gold to violet. Proteins poorly resolved at pH 7 on the IEF gel (Fig. 2) are separated into five visible components in the 2-D gel. Differences in intensity of the spots may be caused by differences in staining, by more than one gene coding for a single protein, or by different rates of synthesis of different zeins.

The zein pattern of inbred A619 is shown in Fig. 3. Differences occur between the patterns of A619 and A632(*Ht*) zeins. Spots indicated by the green arrows in A619's pattern are absent in the pattern of A632(*Ht*), and spots indicated in blue in A632(*Ht*) are absent in A619. Some zeins are common to both inbreds.

The 2-D pattern of the zeins extracted from the hybrid produced by crossing A632(*Ht*) as female with A619 as pollen donor is shown in the center of Fig. 3. All of the zein spots present in extracts from A632(*Ht*) and A619 are present in the pattern of the hybrid's zein. Generally, the unique contributions of the female parent (blue arrows) are about twice as concentrated as protein contributions that are derived only from the male parent (green arrows). The quantity of these proteins appears to be controlled by the dosage of the gene coding for it. Corn endosperm tissue is triploid, having two sets of chromosomes coming from the female and one set from the male parent. Crossing of the two inbreds provides further evidence for equivalence of some of the genes in both parents, because some spots are maintained in the hybrid as single components. The 2-D electrophoresis patterns of zeins from several other hybrids show the same type of relationships to the patterns of zeins derived from their parental inbreds.

Use of 2-D Electrophoresis in Breeding

Many new inbreds were produced by crossing selected older inbreds. Information on derivation of all genotypes was provided by Illinois Foundation Seeds, Inc. (1980). Viotti et al (1982) conclude that the major 20,000- and 22,000-molecular-weight zeins are coded on chromosomes 4, 7, and 10, respectively. Thus, recombinations and possibly mutations affecting zeins will be found in the F_2 generation or backcrosses. In the center of Fig. 4 is the zein pattern of W64A, an important inbred derived from a cross between WF9 and CI187-2, two older established inbreds. In the pattern for W64A, spots derived only from WF9 are indicated by green arrows; zeins contributed only by CI187-2 are labeled with red arrows. Other zeins in the W64A pattern not marked by arrows were coded by genes common to both parental lines and appear in zein patterns of both parental inbreds. Spots 1 and 2 marked by blue arrows appear to occupy the same coordinates in inbreds WF9, W64A, and CI187-2; however, in CI187-2 they are more intense and possibly different in color from the corresponding spots in WF9 and W64A.

Two-dimensional zein patterns of another popular inbred line, Mo17, are compared with its parental lines CI187-2 and C103 in Fig. 5. Reproducibility of extraction and separation by 2-D electrophoresis is shown by the close similarity between the pattern shown for CI187-2 in Fig. 6 (left) and the pattern of zein from another extract from this inbred run at another time (Fig. 5, right). Almost all of the spots in the pattern of Mo17 have counterparts in CI187-2. Two spots present in Mo17 and CI187-2 that are not found in C103 are indicated by arrows without tails. A spot indicated by an arrow with a short tail in CI187-2 is absent in Mo17 and C103. However, another spot in Mo17 indicated by an arrow with a long tail does not appear in either CI187-2 or C103. This zein may have resulted from mutation or recombination either in the parent lines or in the derivatives of their cross. Mo17 apparently inherited all three pairs of CI187-2 chromosomes coding for zeins. Other recombinations must have occurred, inasmuch as phenotypic characteristics differentiate Mo17 from CI187-2.

Relationships of Inbreds

Before the development of hybrid corns, about five or six elite dent varieties became important in the United States because they possessed desirable traits. They served as breeding stocks from which the first inbred varieties were selected (Brown and Goodman 1976, Jugenheimer 1976). Nucca et al (1978) have related a large number of inbreds to these five breeding lines. In Fig. 6, zein patterns from inbreds derived from three of these breeding groups are compared. N-28 is a line derived from the Stiff Stalk Synthetic (SSS) breeding group; H55 is from the Illinois High Yield (HY) variety; and Ohio (Oh) 43 was selected from a cross of Oh 40B (a Lancaster Sure Crop derivative) and W8. Zeins from representatives of two other breeding groups, WF9, which is derived from Reid's Yellow Dent, and C103, a Lancaster Sure Crop derivative, are illustrated in Figs. 5 and 6, respectively. The marked differences indicate that zeins vary most among inbred corns derived from diverse germ plasms.

AI's were calculated for 10 representative inbred corns to determine whether relationships could be determined among different inbreds by analysis of their zeins. Analysis of all the grids recording the component zeins of these 10 inbreds indicated a total of 78 different zeins. Each inbred corn had 25–40 zeins. Based on the calculated AI's, a dendrograph was drawn that related the different inbreds shown in Fig. 7. The relationships in this dendrograph are generally consistent with the classification of inbreds according to the breeding groups of the parents. For example, Nucca et al (1978) classify W64A in the WF9 breeding group, but, as shown in this dendrograph, the agreement of the pattern with its parent C103 line is nearly equivalent to that of the agreement with WF9. Although Nucca et al (1978) regard Mo17 as related to the C103 breeding group, 2-D electrophoresis indicates that its zein components are derived from its CI187-2 parents (Fig. 5). CI187-2 is derived from Krug's Yellow Dent, an old Corn Belt elite variety. Mo17 and CI187-2 share many zein components with N28, a selection from Stiff Stalk Synthetic. Thus, in the dendrograph Mo17 is related to N28 as well as C103.

CONCLUSION

Two-dimensional electrophoresis of zein extracts by IEF followed by PAGE in aluminum lactate buffer with urea gives high resolution of zein proteins. It further emphasizes the heterogeneity of zein and the large number of genes that must code for the different polypeptides. All inbreds we investigated by this procedure differed in their zein compositions. Zeins from hybrid

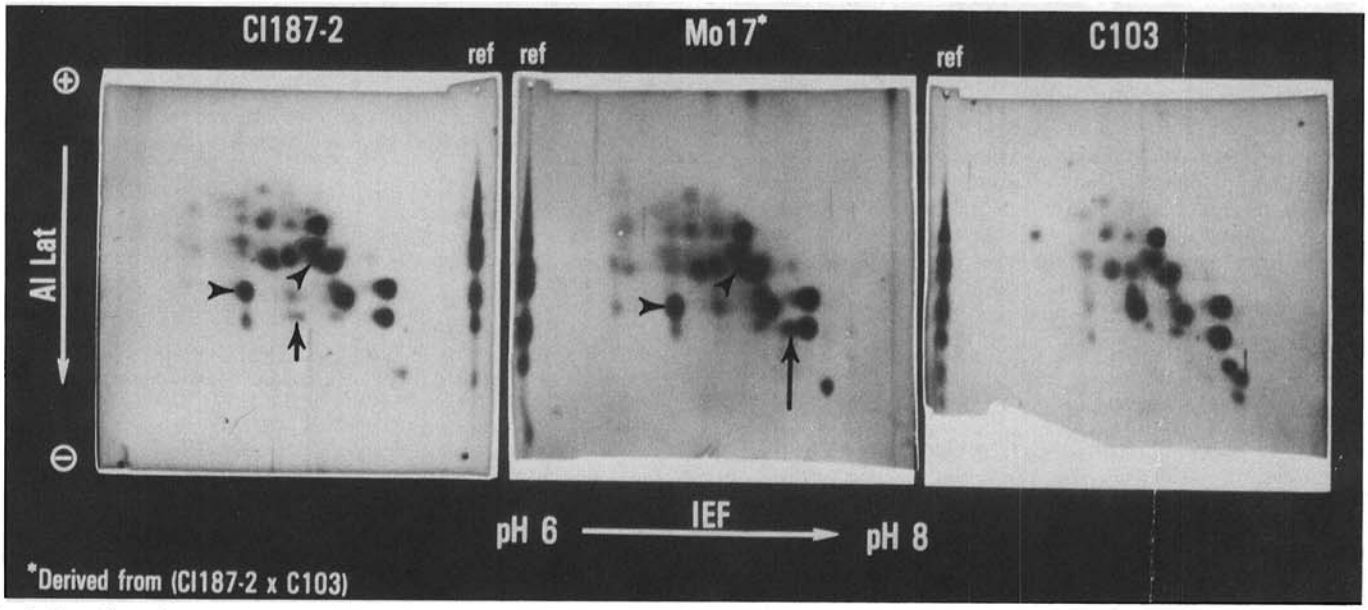

Fig. 5. Two-dimensional electrophoretic patterns of zeins from CI187-2, Mo17, and C103. Mo17 was selected from F_2 generation of a cross between CI187-2 and C103. Arrows without tails indicate proteins common to CI187-2 and Mo17 but not C103. Arrow with short tail indicates protein unique to CI187-2, and arrow with long tail indicates protein unique to Mo17.

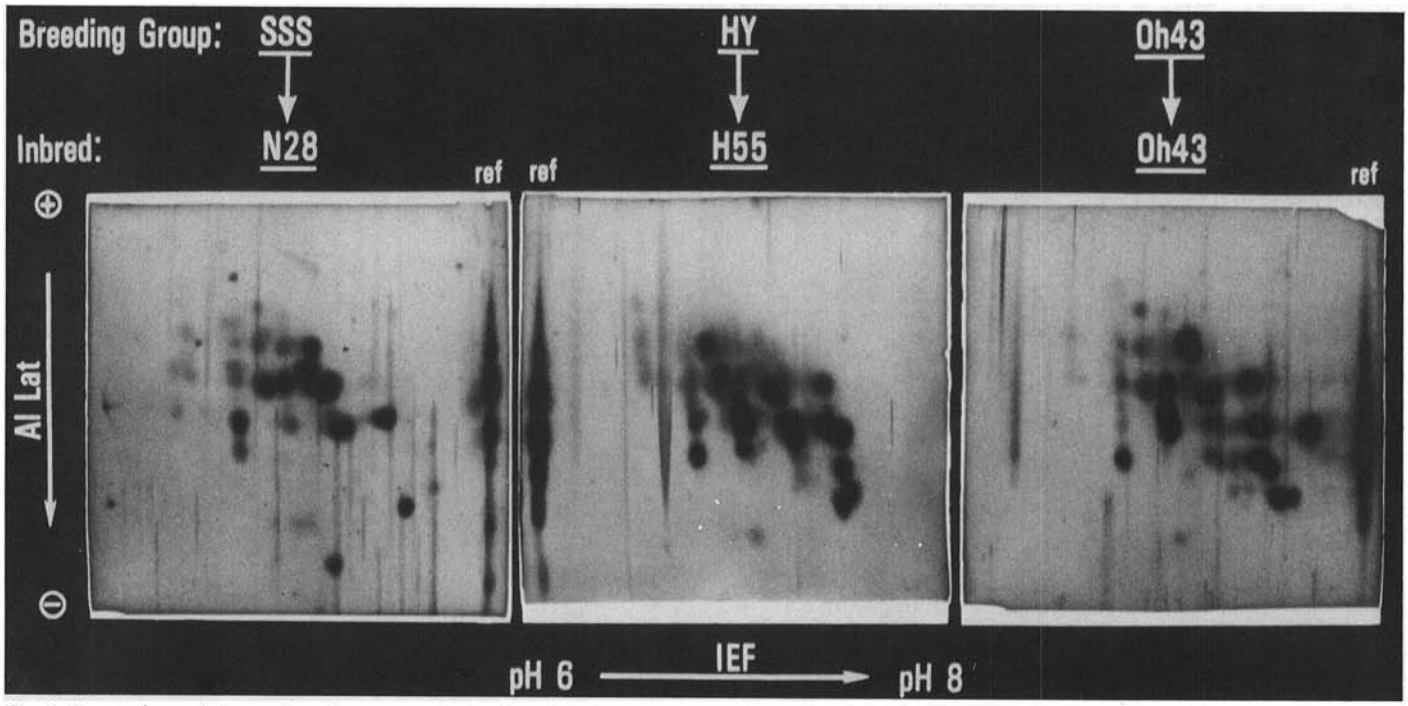

Fig. 6. Comparison of electrophoretic patterns of zeins from inbreds N28, H55, and Oh43 representing breeding groups SSS, HY (Illinois High Yield) and Oh43, respectively.

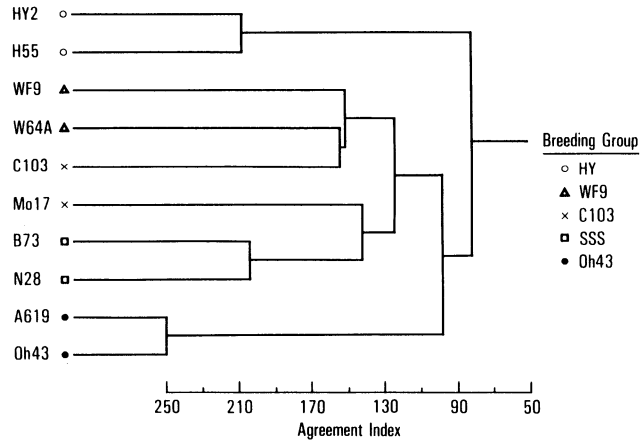

Fig. 7. Dendrograph relating corn inbreds based on calculated Agreement Index of zein patterns.

corns contained proteins from both parents, but the zeins from the female parent were present in about twice the amount as that from the male. It is possible that F_2 selections from crosses of two inbreds can result in retention of zeins from only one parent because the major zeins are coded on only three different chromosomes; for example, Mo17 has a zein pattern like one parent CI187-2. Thus, zein analysis is helpful in identifying inbreds, but it may not be infallible. Perhaps use of other protein fractions also may extend the capability of electrophoresis in identification of corn inbreds.

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