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Heterogeneity of Storage Proteins in Maize

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Abstract. The extensive charge heterogeneity of maize (*Zea mays* L.) zeins observed in isoelectric focusing (IEF) (about 15 bands with pI's in the pH range 6–9) has been found to be independent of extraction procedures or of endosperm development. Zeins do not stain for glycoproteins and exhibit only one lipoprotein component, with pI 3, representing 3–5% of the total protein.

Zeins are very resistant to in vitro deamidation, at both acidic and alkaline pH, at high temperatures, and for rather prolonged times. On the basis of the zein content in acidic and basic amino acids, and of the respective pI's exhibited in IEF (mostly in the pH range 7–8) it has been calculated that at least 90% of the glutamic and aspartic acids ($\sim$52 residues out of a total of $\sim$190) are present as asparagine and glutamine.

Amino acid analysis of zein fractions isolated by preparative IEF has demonstrated changes in the composition of 18 amino acid residues. However, since these changes affect only neutral and hydrophobic residues, it is concluded that the observed zein heterogeneity is partly based on in vivo deamidation of glutamine and asparagine and partly to spot mutations in some of the genes responsible for zein synthesis.

Key words: Protein – Storage protein – *Zea* – Zein.

Introduction

On the basis of their solubility, cereal proteins are classified into three classes: water soluble (albumins and globulins), alcohol soluble (prolamines), and alkali soluble (glutelins) (Osborne and Mendel, 1914). Zein, the prolamine from maize endosperm, accounts for 50–60% of the total proteins. It is synthesized by polysomes bound to specific regions of the endoplasmic reticulum (RER) and accumulated in the form of granules or protein bodies within cisternae (Khoo and Wolf, 1970; Burr and Burr, 1976). Zein is heavily deficient in the essential amino acids lysine and tryptophan, and rich in glutamic acid, proline, leucine, and alanine (Wall, 1964).

Little is known about the molecular properties of zein. Preliminary data from PAGE and IEF have shown a moderate degree of heterogeneity in the zein fraction (Sodek and Wilson, 1971; Paulis et al., 1975; Misra et al., 1975; Correnti and Solari, 1972). Recently, with SDS electrophoresis and with IEF (Soave et al., 1975; Gianazza et al., 1976) we have been able to demonstrate a size and charge heterogeneity in zeins from normal and *o2* maizes. Pooled zeins (i.e., Z_1 and Z_2) are resolved by SDS electrophoresis into four polypeptide bands: two with rather close mol wts 23,000 (Z23) and 21,000 daltons (Z21), one of 13,500 daltons (Z13.5), and another of 9600 daltons (Z9.6). The heavier bands are always the most abundant polypeptides (more than 70% of the total) (Gianazza et al., 1976). While the size heterogeneity is rather restricted, the charge heterogeneity is quite extensive. Zeins are in fact usually resolved, by IEF, into at least 15 components, having pIs in the pH range 5–9, both in normal and *o2* maizes. Furthermore, the IEF components are quantitatively and qualitatively different in various inbred lines of maize (Gianazza et al., 1976; Gentinetta et al., 1975).

In the present paper we report further data on the origin of charge heterogeneity of maize zeins.

Abbreviations: A = absorbance; Bis = N,N′-methylene bisacrylamide; IEF = isoelectric focusing; 2-ME = 2-mercaptoethanol; mol wt = molecular weight; *o2* = opaque-2; PAGE = polyacrylamide gel electrophoresis; pI = isoelectric point; PAS = periodic acid-Schiff stain; SDS = sodium dodecyl sulphate; TCA = trichloroacetic acid; TEMED = N,N,N′,N′-tetramethyl ethylene diamine; Z_1 = zein extracted with 55% isopropanol; Z_2 = zein extracted with 55% isopropanol and 0.6% 2-ME; Z 9.6 = zein of mol wt 9600; Z 13.5 = zein of mol wt 13,500; Z 21 = zein of mol wt 21,000; Z23 = zein of mol wt 23,000

Materials and Methods

Ampholine carrier ampholytes in the pH ranges 6–8, 7–9, and 3.5–10 were purchased from LKB Produkter AB, Bromma, Sweden. Acrylamide, Bis, and sorbitol were from Merk-Schu-

chardt, Munich, Germany. Acrylamide was recrystallized from chloroform and Bis from acetone, as described by Loening (1967). Coomassie Brilliant Blue R-250 was from Serva, Heidelberg, Germany. Riboflavin, ammonium persulphate, and TEMED were obtained from Bio Rad Lab., Richmond, Cal. Sudan Black and the Schiff stain for glycoproteins were from Carlo Erba, Milano, Italy.

Collection of Maize

Several inbred lines of maize and some of their respective homozigous mutants *o2*, produced at the Istituto Sperimentale per la Cerealicultura (Bergamo, Italy) were collected during the summer 1975. Only the endosperm, separated by dissection from embryo and pericarp, was used for study.

Protein Extraction

We used essentially the sequential extraction procedure of Landry and Moureaux (1970), with minor modifications (Soave et al., 1975; Gianazza et al., 1976).

Isolation of Protein Bodies

This was done as described by Christianson et al. (1969) except that the homogenate was layered on a discontinuous sucrose gradient (10 ml 72% sucrose, 20 ml 40% sucrose, and 10 ml 15% sucrose). After centrifugation for 40 min at 25,000 rpm in the SW27 rotor, protein bodies were collected at the 72% sucrose layer surface. The material was diluted two times with 0.1 M phosphate buffer, pH 7.3, and centrifuged 10 min at 10,000 g to pellet the protein bodies. The pellet was extracted two times with 2-ME-isopropanol solvent; the nitrogen content of the extract accounted for more than 90% of the total protein present and it corresponds to zein material as judged by IEF and SDS-PAGE analysis.

Preparative IEF

This was done as described in the LKB 8100 Instruction Manual I-8100-E04. We used the LKB 8100-2 Ampholine electrofocusing column, applying a total sample load of 220 mg zein. The solutions were as follows:

Dense solution: 100 g sorbitol, 63 g urea, 2.5 ml Ampholine pH 6–8, 2.5 ml Ampholine pH 7–9, 0.5 ml Ampholine pH 3.5–10 and 110 mg zein to a final volume of 200 ml;

Light solution: 10 mg sorbitol, 63 g urea, Ampholine as above, and 110 mg zein to a final volume of 200 ml;

Cathode solution: 48 g sorbitol, 30 g urea, and 20 ml of 1N NaOH to 80 ml final volume;

Anode solution: 7 g urea and 6 ml of 1 M H_3PO_4 to 40 ml final volume. The anode was uppermost. The column was run for two days at 10° C at an initial wattage of 6 W (500 V, 12 mA) and a final wattage of 4 W (1000 V, 4 mA) using an ISCO model 492 constant wattage power supply. Since most of the bands were precipitated at their pI, they were eluted by suction from the column top, with the aid of a peristaltic pump.

Amino Acid Analysis

The fractions eluted from the preparative IEF column were extensively dialyzed for 7 days first against 0.1 M NaCl and then against distilled water. Complete Ampholine removal was measured by ninhydrin assay on concentrated aliquots of the solution surrounding the dialysis bag. About 0.5 mg of dialyzed and lyophilized protein was then suspended in 2 ml of 5.7*N* HCl, twice-distilled on quartz. Norleucine (0.03 μM) was added as internal standard to each sample. After hydrolysis (24 h at 110° C) the samples were carried to dryness and appropriate amounts were loaded in a Beckmann Multichrom 4255 analyzer, dissolved in the recommended pH 2.2 buffer. About 98% sample recovery was obtained by this procedure, as judged from norleucine recovery. Automatic peak integration was obtained with a digital PDP8/e computer.

Analytical IEF

IEF was performed in gel slabs by the method of Righetti and Righetti (1975) in an LKB Multiphor apparatus. The gel slabs contained 5% acrylamide (the ratio acrylamide/Bis being 25/1), 2% Ampholine in the pH range 6–9 and 6 M urea. The samples were dissolved in 6 M urea, 10 mM Tris-glycine, pH 8.5, and 2%2-ME and applied to small pockets precast in the gel, containing up to a volume of 30 μl. The samples were applied at the anodic side, to avoid precipitation and aggregation occurring near the cathode, in amounts of 100–200 μg.

IEF was run for 3 h at 4° C with a constant wattage of 13–15 W. For detection of the protein bands, the gel was fixed in 10% TCA for 2 h and the opaque zein bands photographed against a black background with side illumination. Densitometry of TCA-precipitated bands at 450 nm has demonstrated linearity up to 2 A. Alternatively, the TCA-precipitated bands were stained with Coomassie Blue R-250, as described by Righetti and Drysdale (1974). Densitometry of the Coomassie-stained bands has demonstrated linearity up to 3 A.

pI Determinations

Recently, instead of performing pH measurements in gel eluates, as described by Righetti and Drysdale (1971), we have performed them in situ, using an iridium-needle electrode. The gel is scanned at regular intervals (5 mm) with the iridium electrode, while a 1–2 mm hole is punched at each site of pH measurement with the aid of a gel puncher. Readings of electromotive force are then converted into pH units by means of an appropriate calibration scale (P.G. Righetti, E. Gianazza, S. Bordi and G. Papeschi, unpublished).

Specific Stains

The PAS reaction for glycoproteins was developed according to Hebert and Strobbel (1974). Lipoprotein stain with Sudan black has been performed as described by Prat et al. (1969).

Results

Zein Heterogeneity and Extraction Procedure

Zein synthesis begins in endosperm cells around 15 days after pollination and proceeds up to about 40 days (Soave et al., 1975). We extracted zeins with the 2-ME-isopropanol solvent from total endosperm or from protein bodies at two stages of development. The results are summarized in Figure 1. It can be seen that the heterogeneity of zeins extracted from total endosperms is not linked to maturation, since

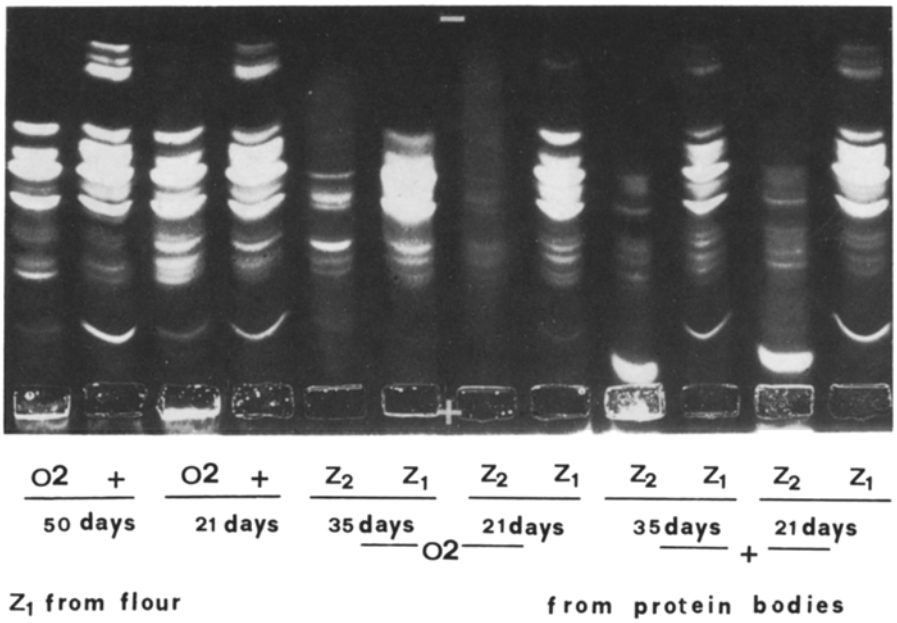

Fig. 1. IEF of zeins from different sources. Conditions: 5% acrylamide gel, 2% Ampholine pH 6–9, 6 M urea; $2^1/_2$ h run at 13 W and 4° C. Gel fixed in 10% TCA for 2 h and photographed against a black background with side illumination. Sample load: 150 μg for Z_1 and 50 μg for Z_2. Anodic application in pockets. Samples: the first four to the left are Z_1 samples isolated from the flour of inbred line W64A (+) or its *o2* mutant (*o2*) either at maturation or at 21 days deveopment. Samples 5–12: are, alternately, Z_2 and Z_1 isolated from protein bodies of *o2* at 35 and 21 days (samples 5–8) or from protein bodies of the normal (+) line W64A at 35 days and 21 days development (samples 9–12)

all the components are present from the onset of zein synthesis and there is no appearance of new nor suppression of pre-existing protein bands. Moreover, the same band distribution is observed when Z_1 and Z_2 are extracted from isolated protein bodies, demonstrating that all the bands resolved by IEF are indeed zeins, since the protein bodies are essentially zein chains surrounded by membranes.

As to the composition of Z_1 as compared to Z_2, it can be seen in Figure 1 that these two zein fractions present a common group of central bands, while Z_2, also exhibits additional components of lower pIs, and Z_1 shows some higher pI bands. This is consistent with our previous findings (Gianazza et al., 1976) that Z_1 consists essentially of a mixed population of Z23 and Z21 chains, while Z_2 is enriched in Z21 as compared to Z23 chains. Moreover, Z_2 contains an additional population of Z13.5 and Z9.6 chains, which focus in the acidic region. It can also be seen in Figure 1 that the *o2* maize exhibits a similar zein polydispersity as normal lines, except for the disappearance of the three most alkaline bands, which represent part of the population of the Z23 chains. This agrees with our previous findings (Gianazza et al., 1976) that the *o2* gene represses the overall zein synthesis but particularly inhibits the synthesis of Z23 chains as compared to Z21, Z13.5, and Z9.6 chains.

Prosthetic Groups

The presence of prosthetic groups, such as sugar or lipid moieties in glyco- or lipoproteins, could be responsible for the heterogeneity observed in zeins. This is particularly worrisome with glycoproteins, since loss of a few sugar residues during purification often results in microheterogeneity that does not reflect the actual protein synthetic activity of the cell (Goldstone and Koening, 1974; Needelman et al., 1975). Zeins purified from inbred lines PA36 and N3811 were subjected to IEF and then stained with the periodic acid-fucsin reagent (Hebert and Strobbel, 1974). No sugar moiety was detected in any of the zein bands, not even in the most acid components. The same experiments were repeated and then the gels stained with Sudan Black for lipoproteins, according to Prat et al. (1969). In this last case a rather acidic protein, having pI ~ 3, and representing approximately 3–5% of the total zein population, was found to stain intensively for lipoproteins (see Fig. 2). This lipoprotein contains a carotenoid covalently bound to the polypeptide backbone and was found to be a component of the membrane that envelops the zein protein bodies in the endosperm. The lipid moiety must be strongly bound to the polypeptide chain, since it is not released by alcohol, Triton X-100, or urea treatment.

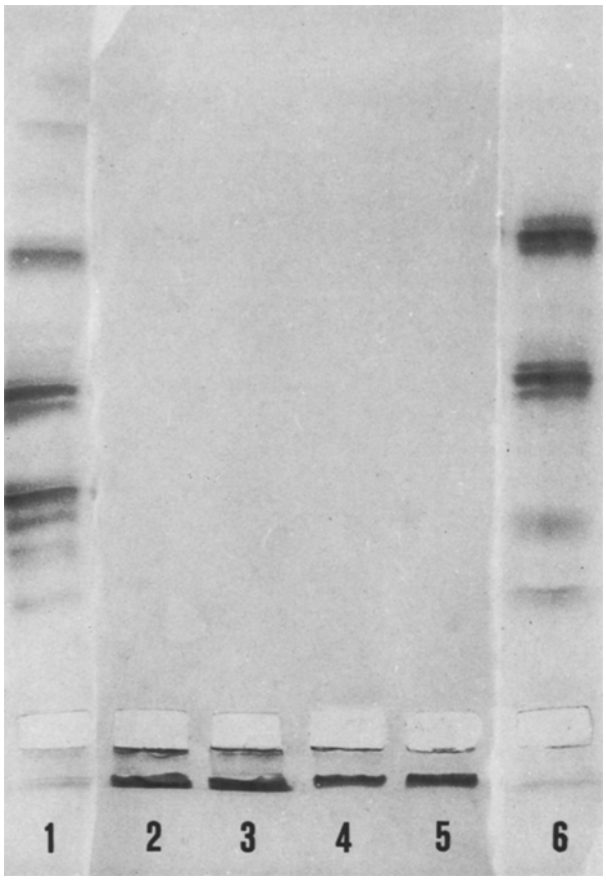

Fig. 2. Lipoprotein stain. IEF as in Figure 1. The two side strips were stained with Coomassie Brilliant Blue R-250, the central part with Sudan Black. Samples: 1–3: Z_1+Z_2 from the inbred line PA36 at maturation; 4–6: Z_2 from the inbred N3811 at maturation

Deamidation as a Source of Charge Heterogeneity

Is is known that most of the aspartic and glutamic acid residues in zein are not present as free acids, but as glutamine and asparagine residues (Wall, 1964). Thus, a partial deamidation of glutamine and asparagine residues could be most likely responsible for the zein heterogeneity observed in IEF. To check this possibility, we have tried chemical deamidation of zeins in vitro. Zeins, extracted from the inbred line N3811, were made 10 mM in formate buffer, pH 3.0, and incubated for up to 8 h at 45° C. The results are shown in Figure 3. It can be seen that the IEF pattern is unaltered, thus indicating that, at least under these conditons, no deamidation takes place. As a further check, we also tried alkaline deamidation. To this purpose, zeins extracted from the inbred line N3811 were incubated either at pH 8.7, or at pH 11, at different temperatures (0°, 20°, or 50° C) for 22 h. As shown in Figure 4, the corresponding IEF is practically unaltered under the various treatments except under very drastic conditions (pH 11, 50° C). Even under these extreme conditions the IEF pattern shift (as indicated by arrows in Fig. 4) is very small and is manifested only at the level of minor components, representing approximately 5% of the entire zein population. Thus it appears that in vitro deamidation, during zein purification and extraction, is a very unlikely event. If deamidation is responsible for zein heterogeneity as determined by IEF, this must be a cellular event, occurring in the cell cytoplasm during zein synthesis and/or subsequent packaging into protein bodies.

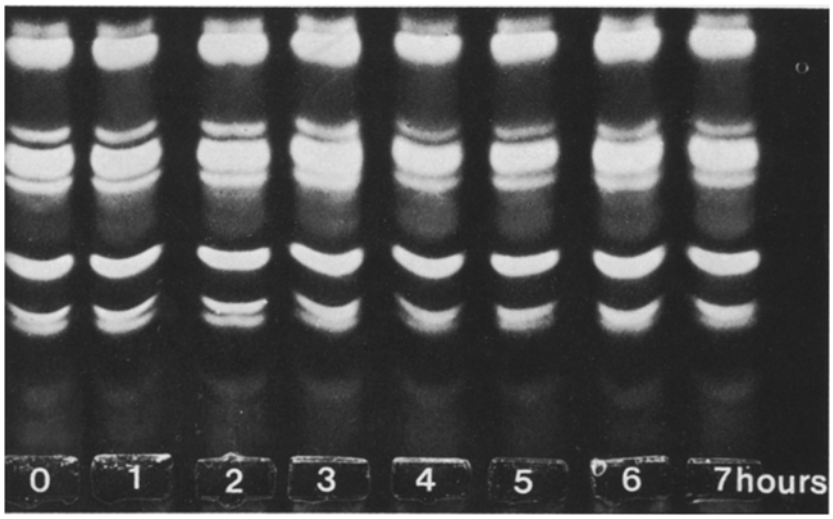

Fig. 3. Acid deamidation. IEF as in Figure 1. Pooled zeins (Z_1+Z_2) from inbred line N3811 were made 10 mM in formate buffer, pH 3.0, and incubated at 45° C. Samples: O: control, untreated; 1–7: samples incubated at pH 3.0 for, respectively, 1, 2, 3, 4, 5, 6, and 7 hours

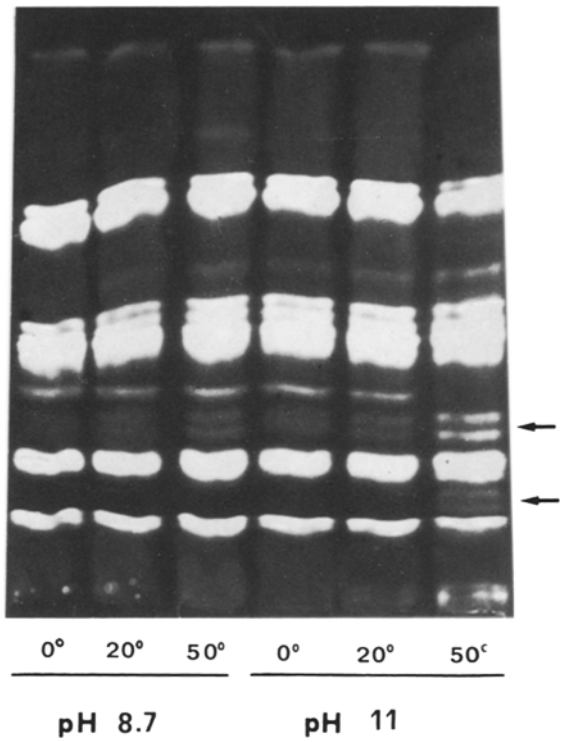

Fig. 4. Alkaline deamidation. IEF as in Figure 1. Samples: pooled zeins (Z_1+Z_2) from inbred line N3811. 1–3 were incubated in 10 mM Tris-glycine, pH 8.7, and 2-ME-isopropanol solvent, for 22 h at 0°, 20°, and 50° C; 4–6 were incubated in 10 mM borate buffer, pH 11, and 2-ME-isopropanol solvent for 22 h at 0°, 20°, and 50° C

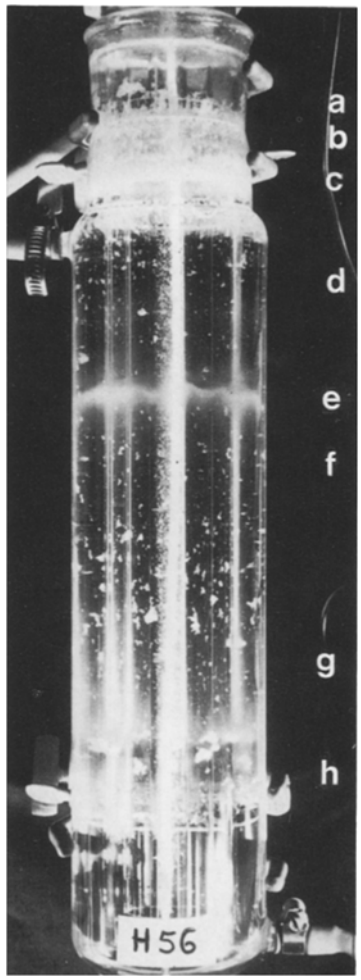

Fig. 5. Preparative IEF: 220 mg of total zein (Z_1+Z_2) from inbred line H56 were loaded on a 5–50% sorbitol gradient in 6 M urea and 1% Ampholine pH 6–9 in the LKB Ampholine 8100-2 column. Focusing at 6 W initial (500 V, 12 mA) and 4 W final (1000 V, 4 mA) at 10° C for 48 h. Peaks A–H, precipitated at their pIs, were eluted by suction from the column top

Amino Acid Analysis of Isolated IEF Components

In order to assess whether the heterogeneity observed in analytical IEF could be due to changes in the primary structure of the different bands, we have run a preparative IEF analysis in a sorbitol density gradient. As shown in Figure 5, much of the same heterogeneity obtained by analytical IEF in gel slabs could be observed also in the preparative column. At least eight protein bands (peaks A–H in Fig. 5), precipitated at their pI, could be distinguished in the column. The various fractions isolated by preparative IEF, and rerun in analytical IEF, show a progressive increase in alkaline components in going from fraction A (low pI components) to fraction H (high pI components) (Fig. 6). The partial contamination of the various fractions is probably due to aggregation caused by hydrophobic interaction together with sedimentation of flocculated material along the sorbitol density gradient column.

Amino acid analysis of the total H56 zein and of the most acidic (A) through the most alkaline (H) bands, has shown a progressive change in some residues. Since H56 zeins are constituted essentially of chains of 23,000 and 21,000 daltons, which correspond to an average of about 190 amino acids per molecule, we have calculated a variation in at least 18 residues in going from peak A to H. Taking into account only those amino acids that would either progressively increase or decrease in the series of peaks A through H, we have calculated positive changes in Ala (+4 residues), Ser (+4 residues), and negative changes in Val (−4 residues) and Tyr (−6 residues) (see Table 1).

The IEF pattern was also unaltered when zeins were focused in 4 M, 6 M, or 8 M urea, in the pres-

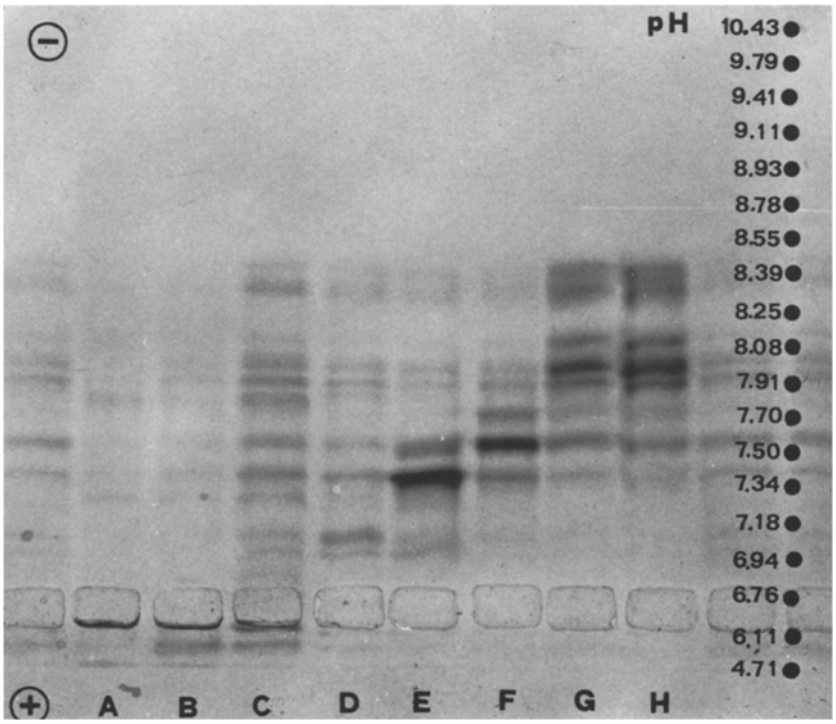

Fig. 6. Analytical IEF. Samples A–H, isolated from preparative IEF (see Fig. 5) were run in gel IEF. Conditions as in Figure 1. Staining with Coomassie Brilliant Blue R-250. First and last samples: control, unfractionated. The pH was determined at 4° C in situ with an iridium electrode. The actual position of pH measurement is the center of each small hole indicated by a black dot to the right of each pH value

Table 1. Amino acid composition of zein fractions from preparative IEF

Amino acid	Number of residues per zein chain. Fractions:						
	A	B	C	D	E	F	G-H
Lys	0.4	1.5	0.6	0.4	0.4	0.6	0.4
His	2.1	2.1	2.3	1.5	2.3	1.9	1.7
Arg	3.0	2.7	2.7	2.3	1.7	1.9	2.3
Asp	9.7	9.3	9.3	8.9	9.1	8.9	8.9
Thr	5.9	5.7	5.9	4.6	4.4	4.6	4.6
Ser	8.4	9.3	9.3	10.1	10.1	12.5	12.5
Glu	36.1	39.9	39.7	42.6	40.7	42.4	40.3
Pro	16.9	19.9	15.6	17.9	19.6	18.4	18.4
Gly	5.5	4.4	4.4	4.2	6.5	4.6	4.0
Ala	21.8	23.7	23.7	23.9	25.5	25.6	26.0
Cys	traces	traces	traces	traces	traces	traces	traces
Val	11.0	9.5	9.3	7.0	7.9	6.8	6.8
Met	2.5	traces	0.9	0.8	0.6	traces	traces
Ile	8.0	7.8	7.2	7.2	6.5	9.3	7.8
Leu	35.0	36.3	35.0	39.1	36.3	37.4	38.2
Tyr	11.8	10.3	10.3	7.2	5.1	5.9	5.9
Phe	14.4	12.9	14.2	11.8	13.5	12.2	12.0

Data from H56 inbred line; fractions A through H run from the most acidic to the most basic zein components. Residue numbers were calculated on the basis of an average mol wt for zein chains of 22×10^3 d, which corresponds to about 190 amino acids per molecule. Each entry is the average of duplicate runs

ence or in the absence of Nonidet P-40 (2%), suggesting that the heterogeneity is not due to different states of aggregation of the zein molecules. The IEF pattern was also not affected by leaving zeins for up to a month dissolved in 8 M urea, suggesting that carbamylation is a very unlikely source of zein polydispersity.

Zein Heterogeneity and Genetic Background

Endosperm cells are triploid since they contain two copies of the maternal and one copy of the paternal chromosomes. We have done reciprocal crosses between inbred maize lines, choosen on the basis of marked differences in their zein IEF patterns. In Figure 7 the patterns of different parental lines and of their two respective reciprocal progenies are compared. Clearly, the F_1 patterns are additive, being constituted by all the bands present in the parental lines. The amount of each component correlates with the gene doses present in each crossing experiment. This suggests that the IEF bands correspond to a system of structural genes acting in an additive way.

We have also demonstrated that, by genetically altering prolamine synthesis, the zein IEF pattern is noticeably affected. Thus, the introduction of the recessive gene *o2* causes a drastic reduction (ca 90%) of the more alkaline IEF components, while leaving practically unaltered (5% reduction) the more acidic bands (see also Fig. 1). Other recessive genes (*opaque-7, floury-2*) have a completely different effect on the zein IEF pattern (Soave et al., 1976).

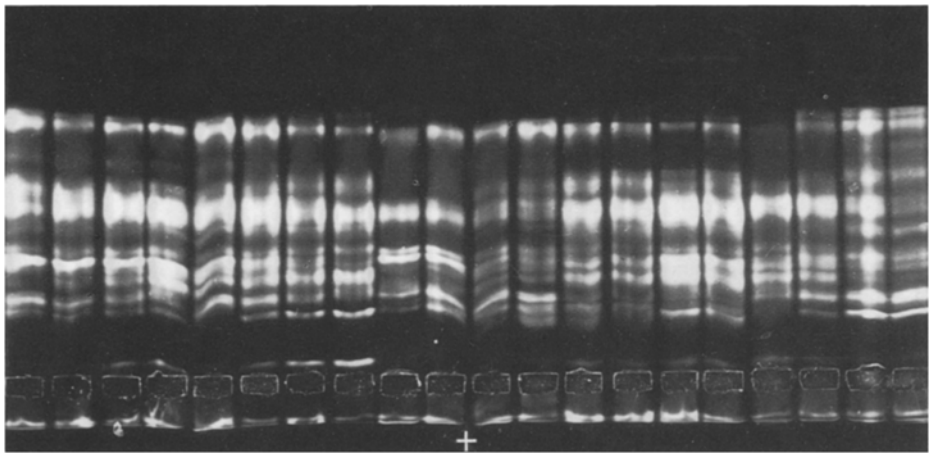

Fig. 7. Genetic crosses. IEF patterns of zeins extracted from normal inbred lines and their two respective reciprocal F_1 progenies. From left to right (by groups of 4): 1st group: K44, K44 × CI44, CI44 × K44, CI44; 2nd group: Va36, Va36 × T222, T222 × Va36, T222; 3rd group: G1315, G1315 × G1330, G1330 × G1315, G1330; 4th group: L1047, L1047 × N28, N28 × L1047, N28; 5th group: Ea2121, Ea2121 × Lo38, Lo38 × Ea2121, Lo38

Discussion

In the present work we have demonstrated that the extensive charge heterogeneity in zein is due to a true cellular event. In fact, this heterogeneity is not due to prosthetic groups, such as a sugar (at least as detectable by PAS staining) or a lipid moiety. Zeins exhibit only a minor lipoprotein component, which is a protein of the membrane surrounding the granules. Zein polydispersity is also not due to different extraction procedures. We can also exclude Ampholine-protein interaction as a possible source of this microheterogeneity, since the same patterns are obtained in gel matrices and in sorbitol density gradients and when deeply altering the protein/Ampholine ratio. It must also be emphasized that, recently, Baumann and Chrambach (1975) and Dean and Messer (1975) demonstrated, by using different proteins with widely differing pIs, that practically no interaction between Ampholine and proteins occurs. Perhaps the strongest evidence against IEF artifacts comes from genetic experiments, which clearly demonstrate that the zein IEF pattern is modulated by the genetic background of each line investigated.

One important question is whether or not part of this heterogeneity could be artifactually elicited by partial deamidation of glutamine and asparagine residues. We have been able to exclude in vitro deamidation, since zeins are quite resistant to deamidation both in acidic and alkaline pH ranges, at high temperatures, and for prolonged times. However, at present, we cannot exclude completely partial deamidation as a cellular event, occurring in the cell cytoplasm perhaps during zein synthesis or during subsequent packaging into granules. It must be stressed, however, that the IEF patterns are kept unmodified at different stages of seed maturation, thus excluding a progressive deamidation.

Some interesting conclusions can be drawn from our amino acid analysis and those reported in the literature (Sodek and Wilson, 1971; Misra et al., 1975; Landry and Moureaux, 1970; Lee et al., 1976). Based on an average mol wt of the two principal zein chains of 22,000 daltons (Burr and Burr, 1976; Misra et al., 1975; Gianazza et al., 1976) we have calculated that zein contains approximately 52 glutamic and aspartic acid residues as acidic groups and 3 arginine, 2 histidine, and less than 1 lysine groups as basic residues. If all the aspartic and glutamic acid residues were present as such, and not amidated, this would lead to a protein having a pI $\sim$3.2. On the contrary, most zeins focus in the pH range 7–8, which means that at least 90% of the glutamic and aspartic acids must be present as glutamine and asparagine residues. Interestingly, by chemical analysis, Wall (1964) reported similar values for Asn and Gln.

By amino acid analysis of peaks A through H, isolated by preparative IEF (see Table 1) we have been able to observe a rather extensive change in amino acid composition, indicating that there is a genetic origin for zein charge heterogeneity. However, since these changes primarily affect neutral or hydrophobic amino acids, they should lead to only minor pI changes in the population of zein molecules. There is also another important source of zein polydispersity. On the basis of our amino acid analysis and those reported in the literature (Sodek and Wilson, 1971; Misra et al., 1975; Landry and Moureaux,

1970; Lee et al., 1976), we have observed that there is one residue, Lys, which is constantly present in stoichiometries of considerably less than 1 mol/mol of zein. Precisely, in any given zein population, only 3 chains out of 5 contain Lys. Clearly, the presence or absence of Lys would greatly contribute to zein heterogeneity. Therefore, we believe that the broad spectrum of pIs observed could be due to random genetic drift of the genes responsible for zein synthesis and, partially, to possible in vivo amidation/deamidation of some Glu and Asp residues.

Similar polydispersity in storage proteins has been found by Wrigley and Shepherd (1973) and by Stegeman et al. (1973). As suggested by these authors, it is possible that these multiple bands could be due to diverging genes deriving from a common ancestral gene. Possibly, it is because the requirements for a storage protein are so broad that there has been a virtual absence of selective pressure against diversification of the genes controlling zein synthesis. In fact, the only functional requirement for zein accumulation is that they maintain their ability to be packaged into granules. We believe that the main forces responsible for these macromolecular aggregates are hydrophobic interactions. In fact, almost 45% of the amino acid residues in zein are hydrophobic, and the acidic residues, which represent approximately 30%, are indeed present as asparagine and glutamine. Only 3% of the total amino acids are basic and there is a virtual absence of methionine and cysteine residues in Z23 and Z21 chains. Therefore, while the extensive mutations we have observed can be tolerated, since they do not affect the overall hydrophobicity of the zein molecules, mutations leading to enrichment in basic amino acids might be less acceptable, since they might disrupt the zein granules. If this is so, this might pose a basic problem in a search for a genetic improvement of maize proteins. It is known, in fact, that zeins have a defect in amino acid composition, since they are deficient in two essential amino acids, lysine and tryptophan (Wall, 1964). If our hypothesis is correct, it should be possible to find mutants richer in tryptophan, but highly improbable to find mutants with higher lysine levels. It is our opinion, in fact, that zeins, to be packaged into granules, cannot tolerate high levels of any of the basic amino acids. In fact, according to the theory of molecular evolution of proteins, as determined by random base changes (Kimura, 1968; Ohta and Kimura, 1971), a zein molecule should contain approximately 16 lysine residues (as opposed to 1), 8 arginine residues (as opposed to three), and 4 histidine residues (as opposed to 2).

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