NOTE

# Isoelectric Focusing of Zein in Agarose[1,2]

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Zein, the major storage protein in maize endosperm, displays much heterogeneity when separated by isoelectric focusing (IEF) in polyacrylamide gels (PAG) (Righetti et al 1977). Some inbred lines reveal up to 15 different zein polypeptides, and a total of 28 different polypeptides has been noted (Valentini et al 1979). IEF has been a useful method for studying gene regulation and linkage relationships of zeins (Valentini et al 1979, Soave et al 1978). It has also been used in taxonomic studies of maize (Nucca et al 1978) and in the evaluation of genetic purity in maize seed stocks (Motto et al 1979).

Recently, IEF in agarose gels was reported to offer advantages over IEF in PAG (Stromska 1982). Agarose forms a large-pore gel that does not sieve most proteins, requires only nontoxic materials, and can be quickly fixed and stained. This research note presents essential details for rapid extraction of zein and its separation by IEF on agarose, which may lead to wider use of IEF for studies of zein.[3]

## MATERIALS AND METHODS

### Zein Extraction

Corn seeds were obtained from manually pollinated plants grown in a field at Urbana, IL. Isolated endosperm samples were pulverized with a microhammer mill (Paulis and Wall 1979). One hundred milligrams of pulverized endosperm were mixed briefly by a vortex mixer with 500 $\mu$l of 55% (v/v) 2-propanol/$H_2O$ containing 1% 2-mercaptoethanol (ME) in a 1.5-ml microcentrifuge tube. The 1:5 ratio of endosperm to solvent usually gave extracts that could be applied directly to the gel. The ratio can be changed when mutant endosperms with different zein contents are extracted. Satisfactory extraction was obtained after 30 min, but the mixture was usually held overnight at room temperature. The mixture was then vortexed again, centrifuged at 8,000 $\times$ $g$ in a Beckman Microfuge, and the supernatant was applied directly to the agarose gel. The extract may be stored in the freezer for a few weeks. The zein content of the extract may be determined by the Coomassie blue G dye-binding assay as modified by Read and Northcote (1981), when zein in 2-propanol is used as a standard. Good patterns were obtained by applying about 20 $\mu$g of zein in 2 $\mu$l to a 2 $\times$ 6 mm slot.

Isolated zein (Wilson et al 1981) may be applied either in alcoholic solution or in a sample buffer consisting of 6$M$ urea in 0.05$M$ glycine adjusted to pH 8 with tris-hydroxymethylaminomethane and containing 1% ME.

### Agarose-Urea Gels

The agarose-IEF system was supplied by the Marine Colloids Division of FMC Corporation and by LKB (Gaithersburg, MD). Many of the details for the preparation of agarose gels and the running of agarose-IEF are given by Righetti (1983) and are described in publications supplied by FMC (Saravis and Cook 1979). The key components are IsoGel agarose (essentially free of electroendosmosis), IsoGel Ampholytes, and GelBond support film. The IEF apparatus used was the LKB Multiphor, which takes 125 $\times$ 245 mm film. The specific results obtained require the use of the items mentioned above, except that any flatbed IEF apparatus having electrodes 100 mm apart and providing cooling should be satisfactory.

The gel consists of 1% agarose, 1.6% pH 5.0–8.0 Ampholyte, 0.4% pH 3.5–9.5 Ampholyte, 5$M$ urea, and 0.002$M$ dithiothreitol (DTT). The agarose is melted in a boiling-water bath with about half the final volume of water, then is cooled to 55° C. A stock urea solution (10$M$, stored in the freezer) is warmed to 55° C and added to make a final concentration of 5$M$, the ampholytes are added, the DTT is added as a 0.06$M$ solution, and the final volume is adjusted to the size required by the apparatus—30 ml for gels 115 $\times$ 235 $\times$ 1 mm.

The warm gel solution is poured between two vertical GelBond sheets secured onto glass plates. On one sheet, the hydrophilic surface is exposed, and on the other sheet, the hydrophobic surface is exposed (this sheet is reusable). The sheets are separated by a U spacer cut from rubber sheeting 1 mm thick and 12 mm wide, and the glass plates are clamped together. Gels containing urea must be "cured" overnight in a refrigerator, and may be stored for several days. The plates are separated by gentle manipulation with a thin piece of wood or plastic, and the gel attaches to the hydrophilic side of the GelBond. The gel is blotted with lint-free filter paper (eg, S&S 577) to remove surface liquid and is laid on the focusing apparatus.

### Sample Application

A sample application mask is a flexible plastic sheet 15 mm wide that extends the width of the gel (perpendicular to the current). Slots 2 $\times$ 6 or 2 $\times$ 10 mm hold 2–3 $\mu$l of sample. The underside of the mask is coated with silicone oil to prevent leakage (Radola 1980). Two samples may be matched by applying 1 $\mu$l of each at opposite ends of the 2 $\times$ 10 slots. More than 40 samples may be separated on a 235-mm-wide gel by applying 2-$\mu$l samples containing about 10 $\mu$g of zein to 2 $\times$ 3 mm pieces of glass fiber filter paper laid on the gel. The samples are applied 25 mm from the anode.

### Focusing

The cathode electrolyte was 1$M$ NaOH, and the anode electrolyte was 0.5$M$ acetic acid. The electrolyte wicks were soaked in the electrolytes, blotted to remove excess solution, and laid on the ends of the gels.

Focusing was begun at 200 V (constant voltage) for 15 min, at which time the plastic masks were removed. The power supply was then switched to constant power and adjusted to give a maximum of 20 W (initial voltage about 700). The voltage increased over the next 20 min until it reached 1,100, the maximum allowed by the power supply. The run was stopped when total volt-hours had reached 1,200–1,300 after about 95 min, at which time the power had dropped to 13 W. Water at 10° C was circulated through the apparatus during the run.

After focusing, the gel, still attached to the GelBond, was fixed in 150 ml of a mixture of 5% acetic acid and 3% trichloracetic acid with slow shaking. The fixer was replaced once or twice over a period of 1 hr. The gel was washed briefly with water, then blotted and blown dry with a hair dryer (Stromska 1982, Saravis and Cook

[1] Cooperative investigations of the U.S. Department of Agriculture, Agricultural Research Service, and the Illinois Agricultural Experiment Station, Department of Agronomy, University of Illinois, Urbana.

[2] 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.

[3] Electrophoretic techniques include a large element of art which causes variations in results obtained by different laboratories. The author will welcome correspondence from those who use these methods and who may need additional details to obtain satisfactory results.

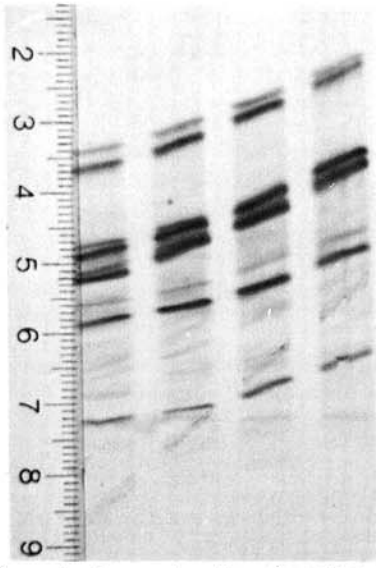

**Fig. 1.** Effect of sample placement on the zein pattern after isoelectric focusing. A sample mask with four 2 × 10 mm slots was placed at an angle (from 86 to 54 mm), and 2 μl of a zein extract from the inbred W64A were placed in each slot. The cathode was at 10 mm, the anode at 110 mm.

1979). The blotted gel, after removal of the filter paper, was washed in the fixer for another hour.

The gel was stained by shaking for 10 min in a solution made by adding 5 ml of 1% Coomassie blue R (Acid Blue 83, CI 42660) in methanol to 100 ml of methanol-acetic acid-water (20:10:70, v/v). The gel was destained for about 10 min in the same solvent, rinsed in 1% acetic acid and then in water, and finally air-dried. Total elapsed time was as little as 5 hr. The dried gel is firmly bound to the GelBond, and can be conveniently stored in a ring binder. The stained bands showed no signs of fading after several months.

After fixation (before or after blotting) the wet gels could be photographed by backlighting against a black background. Stained gels were photographed on a fluorescent light box with a yellow (Wratten 15) filter (Wilson 1979). Polaroid 55 P/N film in an MP-3 camera or Kodak Plus-X film in a 35-mm camera both gave satisfactory results.

## RESULTS AND DISCUSSION

The system outlined here is the result of many trial-and-error experiments, and only the important results will be presented.

Sample preparation is especially easy. The small volume required does not contain enough alcohol to affect the gel. The extracts are not stable for more than three weeks, but surplus

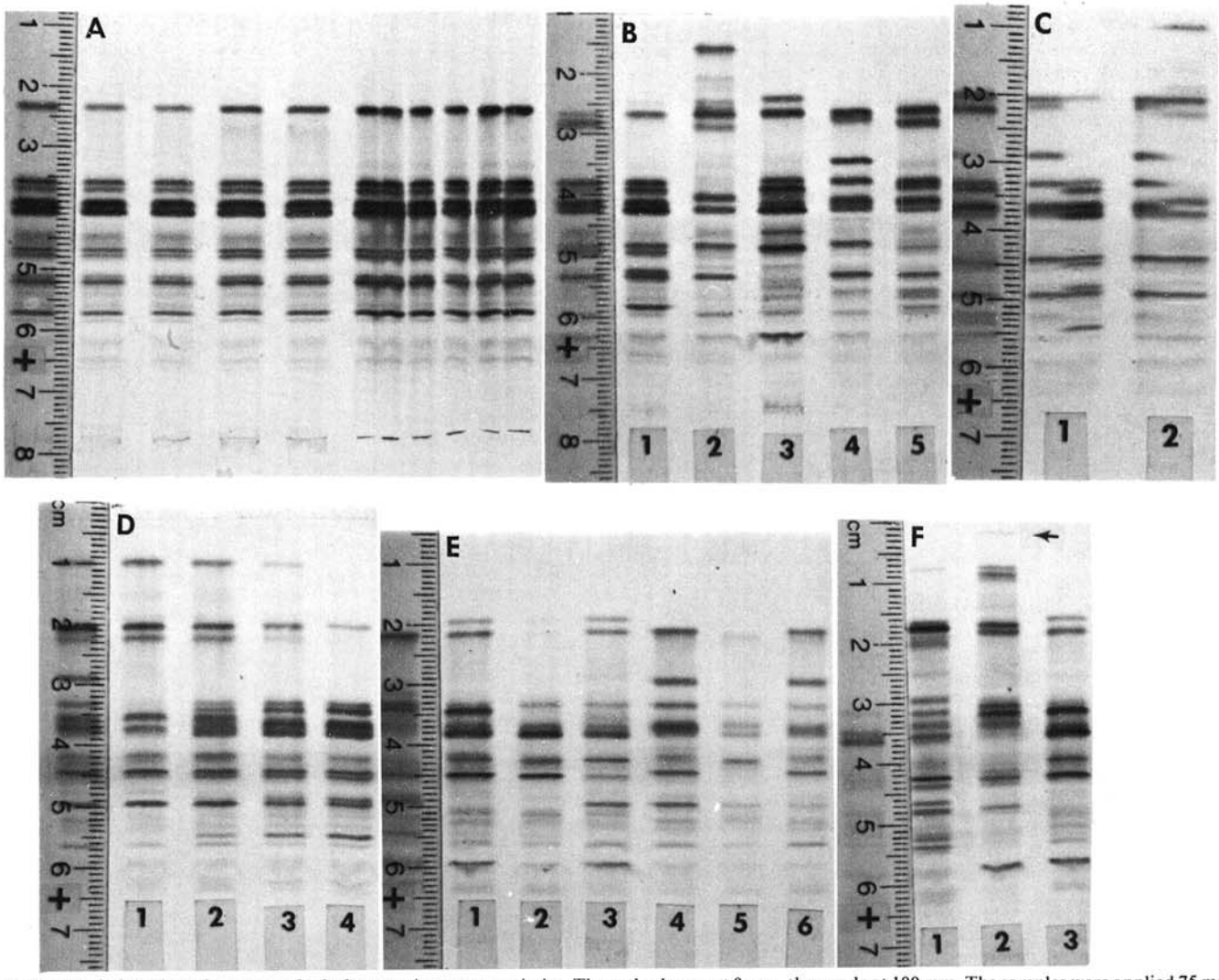


**Fig. 2.** Isoelectric focusing of patterns of zein from various corn varieties. The cathode was at 0 mm, the anode at 100 mm. The samples were applied 75 mm from the cathode. The samples (2 μl) were placed in 2 × 6 mm slots in plastic masks, except as noted. **A,** Ten single endosperm extracts of the inbred Mo17. The six samples on the right side were applied to 2 × 3 mm glass fiber strips, which were laid on the gel. **B,** Inbreds: 1, N28; 2, Oh43; 3, W64A; 4, W22; 5, Va35. **C,** Paired 1-μl extracts of the inbreds: 1, W22 and N28; 2, W22 and Oh 43. Inbreds were applied at opposite ends of 2 × 10 mm slots. **D,** Two inbreds and the reciprocal F1 hybrids: 1, Oh43; 2, Oh43 × N28; 3, N28 × Oh43; 4, N28. **E,** Mutant endosperm extracts: 1–3 W64A, normal, *opaque*-2, and floury-2; 4–6 W22, normal, *opaque*-2, and *opaque*-7. **F,** 1 = *Zea mays* subsp. *mexicana*, var. *huehuetenangensis*, 2 = *Zea diploperennis*, and 3 = *Zea mays* subsp. *mays*, inbred W64A.

pulverized endosperms can be stored for months in the refrigerator and then can be extracted as needed. In samples much smaller than 100 mg, only a portion of a single seed is needed and the rest can be planted. Some differences were noted between samples extracted with and without ME in the alcohol, but the differences disappeared if ME was added to the samples without ME before focusing. Sharper bands and fewer differences caused by apparent oxidation-reduction reactions were seen when the extracts always contained ME and the gels contained DTT. Reduction and alkylation of the proteins might give increased stability and better banding patterns (Wilson et al 1981), but sample preparation would be much more difficult.

The final location of each band depended on the starting position and on the total volt-hours used, but reproducibility was good when these factors were controlled. Band positions shifted when the sample was applied with the mask laid down at an angle (Fig. 1), but the best results were obtained when the starting position was 25 mm from the anode. Focusing for up to 2,400 volt-hours did not improve the separation because there was a slow drift of all bands toward the cathode. pH measurements were not made because the zein polypeptides did not reach equilibrium positions in relation to standard proteins (not shown), so any matching of zein bands with pH readings are arbitrary. However, apparent pH values after IEF in open horizontal slab gels are affected by $CO_2$ absorption (Delincée and Radola 1978), urea, and temperature (Gianazza et al 1977), and the values of pH measurements are thus reduced.

IsoGel agarose was selected because it had satisfactory IEF properties and retained enough strength in the presence of 5*M* urea to remain attached to the GelBond throughout the entire procedure. Other IEF agaroses were tested, but they tended to separate from the GelBond. The GelBond support made handling and storing of the gels very easy.

The staining reaction was fast and gave intensely stained bands, probably because of the thinness of the dried gel. The alcohol content of the staining and destaining solutions was a compromise between the high alcohol concentration needed for good dye solubility and the low concentration needed to keep zein fixed (Wilson 1979). Methods for IEF of zein in PAG require more than 100 $\mu$g of zein per sample (Paulis and Wall 1979, Righetti et al 1977, Soave et al 1978, Wilson et al 1981), compared to the 20 $\mu$g or less needed for this technique.

Figure 2 shows several applications of the procedure. Figure 2A demonstrates the reproducibility of the patterns among zeins extracted from separate seeds of one inbred line, as well as the close packing obtained when glass-fiber paper strips were used to apply the sample. The 10 seeds came from five ears harvested in two years. Figure 2B shows the variable patterns obtained from five inbreds. Figure 2C shows that closely spaced bands in two samples can be matched (or not matched) by applying the samples at opposite ends of a 2 × 10 mm sample slot. This technique made it possible to detect a total of 33 different bands among the five inbreds shown in Fig. 2B. Each inbred line revealed from 14 to 17 bands, 7–10 of which were prominent. Only three bands occurred in all five inbreds, whereas 19 stained intensely in at least one inbred. These inbreds make good standards for band identification.

Endosperms contain three sets of chromosomes. Figure 2D shows that the inbreds Oh43 (lane 1) and N28 (lane 4) differ by several bands (eg, 10 and 34 mm), which occur with intermediate intensities in the reciprocal hybrids containing one (lane 2) or two (lane 3) doses of N28 genes. Figure 2E shows that several zein bands disappeared from the *opaque*-2 versions of the inbreds W64A (lane 2) or W22 (lane 5), whereas the floury-2 (lane 3) or *opaque*-7 (lane 6) versions were more like the normal patterns. Zeins extracted from the wild corn relative, teosinte, are shown in Fig. 2F. Several of the teosinte bands corresponded with the bands present in a corn belt inbred (lane 3), but a number of new bands are seen, including the most basic band yet found, which is marked with an arrow.

The patterns were reproducible from run to run, but given bands appeared at slightly different places and with slightly different spacings, so it is essential to run known standards on each gel.

The ease of sample preparation, the nontoxic reagents, the speed of the assay, and the sharpness of the bands recommend agarose IEF for separation of zein polypeptides. Additional polypeptides might be revealed by using sodium dodecyl sulfate PAG electrophoresis for a second dimension (Wilson et al 1981), but this one-dimensional IEF method gives sufficient separation of heterogeneous zein proteins for it to be useful for many purposes.

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## LITERATURE CITED

DELINCÉE, H., and RADOLA, B. J. 1978. Determination of isoelectric points in thin-layer isoelectric focusing: The importance of attaining the steady state and the role of $CO_2$ interference. Anal. Biochem. 90:609.

GIANAZZA, E., RIGHETTI, P. G., BORDI, S., and PAPESCHI, G. 1977. pH determinations in isoelectric focusing with an iridium electrode. Page 173 in: Electrofocusing and Isotachophoresis. B. J. Radola and D. Graesslin, eds. Walter deGruyter, New York.

MOTTO, M., SALAMINI, F., REGGIANI, R., and SOAVE, C. 1979. Evaluation of genetic purity in hybrid corn (*Zea mays* L.) seed production through zein isoelectrophoretic patterns. Maydica 24:223.

NUCCA, R., SOAVE, C., MOTTO, M., and SALAMINI, F. 1978. Taxonomic significance of the zein isoelectric focusing pattern. Maydica 23:239.

PAULIS, J. W., and WALL, J. S. 1979. Note on mill for pulverizing single kernels of cereals for isoelectric focusing. Cereal Chem. 56:497.

RADOLA, B. J. 1980. Ultrathin-layer isoelectric focusing in 50-100 $\mu$m polyacrylamide gels on silanized glass plates or polyester films. Electrophoresis 1:43.

READ, S. M., and NORTHCOTE, D. H. 1981. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. Anal. Biochem. 116:53.

RIGHETTI, P. G. 1983. Isoelectric Focusing: Theory, Methodology and Applications. Elsevier, New York.

RIGHETTI, P. G., GIANAZZA, E., VIOTTI, A., and SOAVE, C. 1977. Heterogeneity of storage proteins in maize. Planta 136:115.

SARAVIS, C. A., and COOK, R. B. 1979. Agarose isoelectrofocusing with IsoGel agarose and GelBond film. Marine Colloids Div., Bioproducts Dept., FMC Corp., Rockland, ME.

SOAVE, C., SUMAN, N., VIOTTI, A., and SALAMINI, F. 1978. Linkage relationships between regulatory and structural gene loci involved in zein synthesis in maize. Theor. Appl. Genet. 52:263.

STROMSKA, D. P. 1982. Isoelectric focusing of tubulin in a new type of charge-balanced agarose. Anal. Biochem. 119:387.

VALENTINI, G., SOAVE, C., and OTTAVIANO, E. 1979. Chromosomal location of zein genes in *Zea mays*. Heredity 42:33.

WILSON, C. M. 1979. Studies and critique of Amido Black 10B, Coomassie Blue R, and fast green FCF as stains for proteins after polyacrylamide gel electrophoresis. Anal. Biochem. 96:263.

WILSON, C. M., SHEWRY, P. R., and MIFLIN, B. J. 1981. Maize endosperm proteins compared by sodium dodecyl sulfate gel electrophoresis and isoelectric focusing. Cereal Chem. 58:275.

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