A Nomenclature for Zein Polypeptides Based on Isoelectric Focusing and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis[1,2]

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ABSTRACT

Isoelectric focusing (IEF) of zein in agarose gels separates zeins from corn endosperms into 30 components from different inbreds. Each inbred exhibits seven to 12 bands useful for comparing inbreds, varieties, and hybrids. A nomenclature based on IEF patterns of six inbreds permits comparisons of the many possible patterns. Closely related inbreds are often very similar, but some differences occur. Some polypeptides are absent in *opaque*-2 (high-lysine) mutants, and others are extracted only in the presence of reducing agents. Single IEF bands were easily removed, complexed with sodium dodecyl sulfate, and analyzed by polyacrylamide gel electrophoresis to indicate estimated molecular masses. A proposed zein nomenclature using IEF and sodium dodecyl sulfate polyacrylamide gel electrophoresis banding patterns is outlined.

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Zein, the major storage protein in corn endosperm, can be separated into size classes by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Wilson et al 1981). Zein appears even more heterogeneous upon isoelectric focusing (IEF) (Righetti et al 1977). IEF in agarose gels is more convenient than in polyacrylamide gels (PAG) and produces sharp separations of many zein polypeptides (Wilson 1984). A nomenclature was developed to identify individual zein IEF bands in agarose (Wilson 1985). Twenty-five corn inbreds contained a total of 25 bands; each inbred had 7–12 bands sufficiently intense to be useful for identification. With one exception, inbreds possessing identical patterns were closely related. The number of useful bands has since increased to over 30, and 40 inbreds have shown distinguishable patterns (Wilson, *unpublished*).

SDS-PAGE is perhaps the most widely used method for analyzing zeins. There have been problems in relating the classical Osborne seed protein fractions to these new methods. The glutelin fraction from corn appears to consist of a mixture of unrelated proteins united only by being in the residue from previous extractions (Shewry and Miflin 1985, Wilson 1983, Wilson et al 1981). It has been suggested that corn seed proteins may be designated as "basic" or "endosperm specific" (Landry and Moureaux 1980) or as storage, metabolic, and structural proteins (Byers et al 1983). The endosperm-specific or storage proteins are associated with endosperm protein bodies (Vitale et al 1982), from which they can be extracted with alcoholic solutions (Miflin et al 1981, Taylor et al 1984). Reducing agents must be added to the alcohol to extract some proteins. This paper will be concerned with four classes of protein soluble in alcoholic solutions with reducing agents, but not with the protein body protein that is also soluble in aqueous solutions (Wilson et al 1981). These proteins are the two traditional zein fractions and two small molecular mass proteins of somewhat different amino acid contents (Shewry et al 1985) that have the prolamin type of amino acid composition. The two smaller zeins are found in a zein-2 extract (Taylor et al 1984) as well as in total extracts made under reducing conditions (Wilson et al 1981).

Gorinstein et al (1983) noted the disagreement in the literature for the apparent molecular masses of the two larger zeins, as determined by SDS-PAGE. I have seen reports for the four zeins with wide ranges: 27–21, 25–18, 19–13.5, and 14–9.6 kDa (references available upon request). It may be most common to refer to the zeins as Z22, Z19, Z15, and Z10 (Shewry and Miflin 1985). Any use of numbers alone may cause confusion, because the same number may be used by different authors to refer to different zeins. I am presenting a nomenclature for zeins separated by SDS-PAGE in which they are called A-, B-, C-, and D-zeins in order of descending size, which remains neutral on the question of which number is correct.

Recently the primary structures of DNAs coding for zeins have been determined (reviewed in Shewry and Miflin 1985). In one example Hu et al (1982) reported molecular masses of about 27 and 23 kDa for A- and B-zeins. Eventually this data should be matched to data on the individual proteins.

IEF and SDS-PAGE can be performed sequentially to produce two-dimensional (2-D) separations of zeins (Wilson et al 1981), and different patterns can be detected in mutants (Burr and Burr 1982). Nevertheless, no generalized comparison system has been reported for zeins because of the difficulty of using precise mass and charge markers. Vitale et al (1980) isolated the individual zeins by IEF in PAG, and then characterized each zein band by SDS-PAGE. Most IEF bands gave single spots upon SDS-PAGE, but some separated into two spots.

The present study demonstrates that closely related inbreds may be characterized by IEF in agarose, that agarose IEF detects changes in zein patterns caused by the *opaque*-2 mutation, that some zeins are extracted by alcohol only when mercaptoethanol (2-ME) is present, and that individual zein bands from agarose IEF gels may be conveniently characterized by SDS-PAGE. These procedures are combined to produce a useful nomenclature system for the zeins.

MATERIALS AND METHODS

Sample Preparation

Zeins were extracted as described previously (Wilson 1984) from inbred corn seeds grown on the Agronomy–Plant Pathology South Farm, Urbana, IL. Mature endosperm was crushed, extracted with 55% (v/v) 2-propanol containing 1 or 2% 2-ME, and centrifuged. IEF patterns were obtained by applying about 20 μg zein (2 μl of extract) to 1 × 7 or 2 × 10 mm slots cut in plastic sample masks.

IEF in Agarose

The agarose-IEF system (Wilson 1984, 1985) used a 0.75 mm layer of 1% IsoGel, 1.6% pH 5–8 (or pH 6–8) Ampholine, 0.4% pH 3.5–9.5 Ampholine, 5*M* urea, and 2 m*M* dithiothreitol on a GelBond sheet. Focusing was performed on an LKB Multiphore apparatus (electrodes 10 cm apart) for a total of 1,300 volt-hours. The gels were fixed, blotted, dried, fixed again, and stained with 1% Coomassie Blue R (CI 42660, obtained from Sigma Chemical Company). After destaining, the gels, still attached to the GelBond sheet, were stored dry at room temperature.

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SDS-PAGE

The procedure of Laemmli (1970), using Tris-glycine buffer with 0.1% SDS, was used to separate zein polypeptides. Gels with 12% acrylamide plus bis-acrylamide and 2.6% crosslinking were cast in a Bio-Rad Protean slab apparatus, forming a separating gel 1.5 mm thick, 14 cm wide, and 12 cm long. Sample slots 4 mm wide were cast in a 4% stacking gel. Electrophoresis proceeded until the bromophenol blue dye front had migrated 11 cm, after which the gels were stained with 0.25% Coomassie Blue R in methanol, acetic acid, and water (40:10:50, v/v) for at least 2 hr. The gels were then washed with a solution of methanol, trichloroacetic acid (TCA), and water (40:10:50) for about 5 min, and were destained by shaking in 12½% TCA. The TCA solution was changed several times until the background was low. The final solution exchange before gels were stored in plastic bags was with 10% acetic acid.

The dry IEF gels and the wet SDS-PAGE gels were scanned at 565 nm with the linear transporter of a Gilford spectrophotometer, and recordings were made with a Hewlett-Packard 3390A reporting-integrator.

IEF samples to be subsequently analyzed by SDS-PAGE were prepared by applying 5 μl of zein extract to 2 × 10 mm slots in the sample mask, more than double the quantity applied for analytical separations. IEF was performed as usual. To remove a sample from the dry agarose gel, a small area was wiped with a wet cotton swab to soften the agarose. A scratch was drawn around the desired band, which could then be peeled off the GelBond and transferred to a plastic microcentrifuge tube (see Fig. 4). The volume of SDS sample buffer added to each fraction was adjusted to the amount of zein in each band, as estimated by stain intensity. More than one sample could be used for weak zein bands or if repeated analyses were to be made. The SDS sample buffer contained pH 8.5 Tris-glycine buffer (0.01*M*), 0.2% SDS, 10% sucrose, and 2% 2-ME. The samples were placed in a boiling water bath for 5 min and then were kept hot until added to the sample slots. A Drummond 25 μl Wiretrol microdispenser fits into the sample slot and can be operated fast enough to prevent the agarose from gelling until dispensed. Unused sample may be stored in the freezer, but the agarose may not gel again after a freeze-thaw cycle. The Coomassie Blue R separates from protein upon electrophoresis and serves as a tracking dye, migrating slightly faster than bromophenol blue.[4]

RESULTS AND DISCUSSION

Isoelectric Focusing

The zein IEF band identification and nomenclature (Wilson 1985) are shown in Figure 1. These six inbreds contain most bands found in common inbreds and were compared to unknown samples for band matching and identification. The numbers approximate the distance (in millimeters) of the bands from the cathode in a typical run; the actual distances vary from run to run, so it is necessary to run standard inbreds on each gel for calibration. Several bands between 10 and 20 mm and between 38 and 60 mm occur in low amounts in some inbreds and are not always seen.

The relatively large number of bands and the sharp separations obtained by agarose-IEF allow many identifications by separation in one dimension only. Typical differences among closely related inbreds are illustrated in Figure 2. Inbred 38-11 (no. 4) is an inbred used in making the Iowa Stiff Stalk Synthetic line, from which many common inbreds were derived, including B14A (no. 2) and N28 (no. 5). B14A was then used as a parent for R801 (no. 1) and A632 (no. 3). Visual observations, and the band-matching procedure in which two samples are placed at opposite ends of a 2 ×10 mm slot (Wilson 1984), reveal that the uppermost bands in R801 (Fig. 2, arrow a) are closer together than the pair of bands in B14A and A632. These are bands 20 and 21, or 21.5, respectively. The gels were scanned, and the spacing was found to average 1.73 mm for R801 and 2.23 mm for B14A. The separation on this particular gel was greater than the 1 or 1.5 mm estimated with a

[4] Electrophoretic techniques include a large element of art which causes variations in results obtained by different laboratories. The author has prepared a set of supplemental instructions which may assist those actually using agarose-IEF for zein assays, and which will be sent upon request.

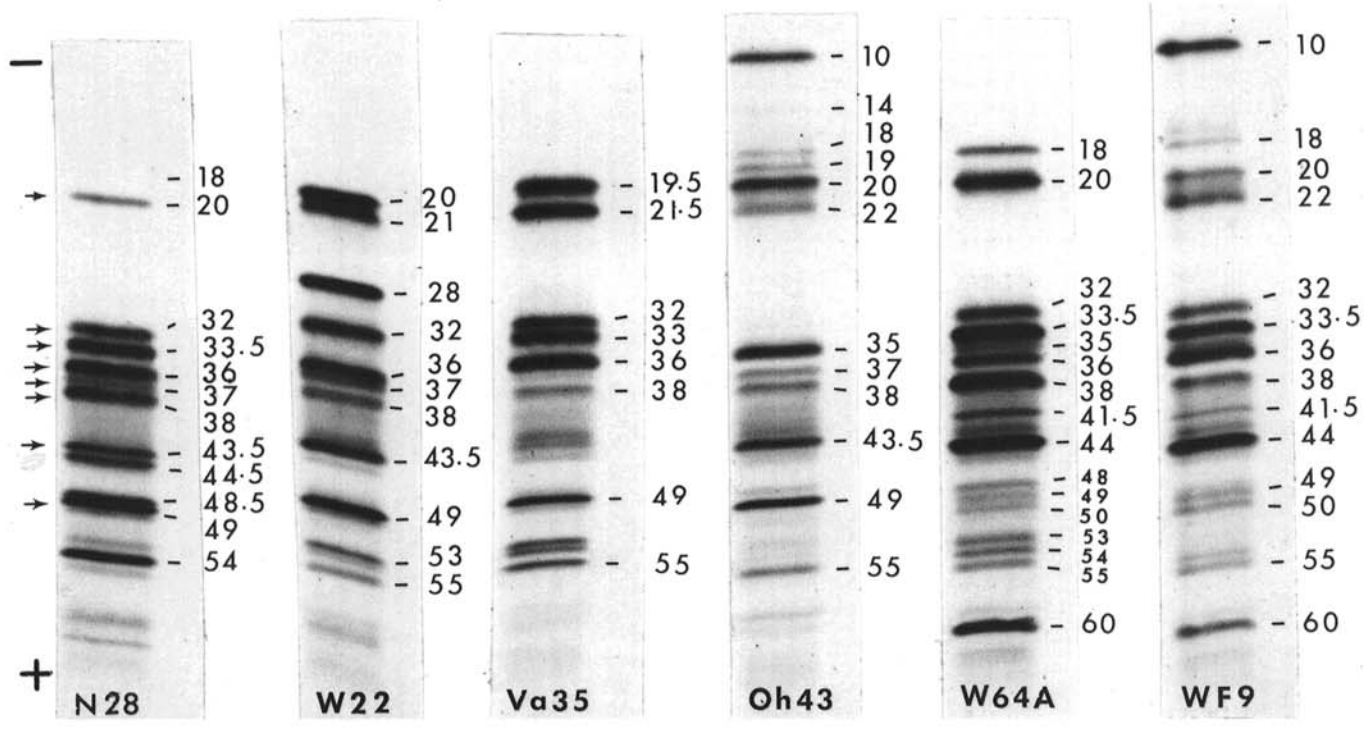

Fig. 1. Zein band numbering system for isoelectric focusing in agarose. The numbers represent distances in millimeters from the cathode of zein bands on a "typical" gel with four parts of pH 5–8 and one part of pH 3.5–9.5 Ampholines. A few bands were not readily visible when photographed. From Wilson (1985).

ruler on the original standard gel, but matching to standard inbreds confirmed the identities. R801 and B14A had dark bands at 49 (Fig. 2, arrow b) that were absent from A632. A632 had a faint band at this position, but the lack of sharpness suggests that it might be a different polypeptide present in a very small amount. Other assays will be required to identify this band. Finally, R801 had band 55 (Fig. 2, arrow c), which was absent in parental line B14A and related A632.

SDS-PAGE Zein Nomenclature

An idealized zein band pattern produced by SDS-PAGE is shown in Figure 3. It is discussed in detail elsewhere (Wilson 1983, and *unpublished*). To avoid the confusion caused by the varying reported molecular masses, the zein polypeptides are identified as A-zein, B-zein, C-zein, and D-zein, listed from the largest (slowest) polypeptide to the smallest. A- and B-zeins are almost the only components found in a zein-1 extract (without 2-ME), whereas the C-and D-zeins are included in a "total" zein extract, made with 2-ME in the alcoholic solvent. A- and B-zeins each separate into two or more bands, but such separations are difficult to reproduce because of minor differences in acrylamide, catalysts, and final gel consistency.

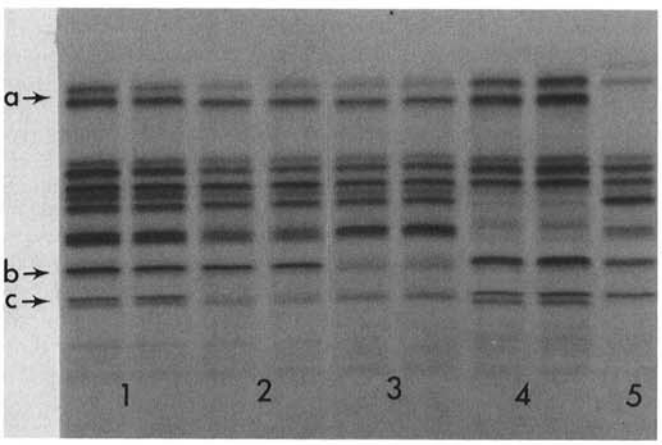

Fig. 2. Isoelectric focusing patterns of zein from five related inbreds. **1**, R801; **2**, B14A; **3**, A632; **4**, 38-11; and **5**, N28. Arrows mark bands discussed in test. (Compare with Fig. 1, N28, for band numbers.)

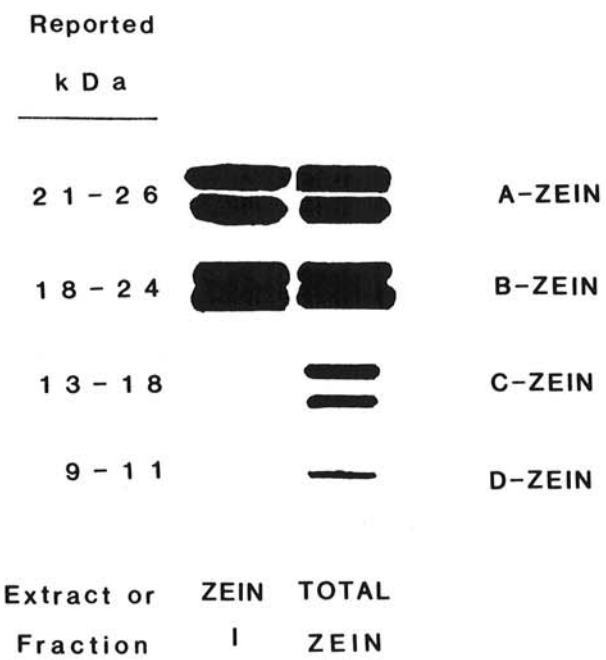

Fig. 3. An idealized nomenclature for zein polypeptides separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Zein-1 is an alcoholic extract made without mercaptoethanol (2-ME) but reduced before running on the gel. Total zein is an extract made with 2-ME in the extracting solvent.

2-D Separation

A 2-D separation of zein polypeptides by IEF and SDS-PAGE is shown in Figure 4. For IEF, each sample slot held about 58 μg of zein instead of the usual 20 μg, so separations were not as good as usual. Bands from six lanes (about 350 μg total zein) were scraped off and combined, and 100–400 μl of SDS sample buffer was added to each. After treatment in a boiling water bath, from 30 to 50 μl was added to sample slots of an SDS-PAGE slab gel. The amounts of zein added ranged from an equivalent of 2.25 μl of the original extract of total zein (26 μg) for major bands 36 and 38 to a maximum of an equivalent of 15 μl (175 μg total zein) for bands 60 and 62 (minor constituents). Varying sample size allowed minor bands to be assayed without overloading major zein bands. The individual samples allow runs to be made with, for example, band 32 samples from several inbreds side-by-side to match estimated molecular masses, or to compare the mobilities of bands 32 and 49 from N28 (not shown).

N28 has a minor band at 37 (Fig. 1) which was not apparent upon overloading (Fig. 4). In another experiment, band 37 was found to be an A-zein, presumably the minor A-zein detected with the major B-zein bands for IEF bands 36 and 38 (Fig. 4). Band 20 is an A-zein slightly larger than band 33.5. Further tests are needed to determine if band 33.5 consists of both A- and B-zeins, or if the minor B-zein is a contaminant. C-zein has been found in band 42.5 in several inbreds, and D-zein has been found in band 55. IEF bands of C- and D-zeins are not always well formed, however, and are less

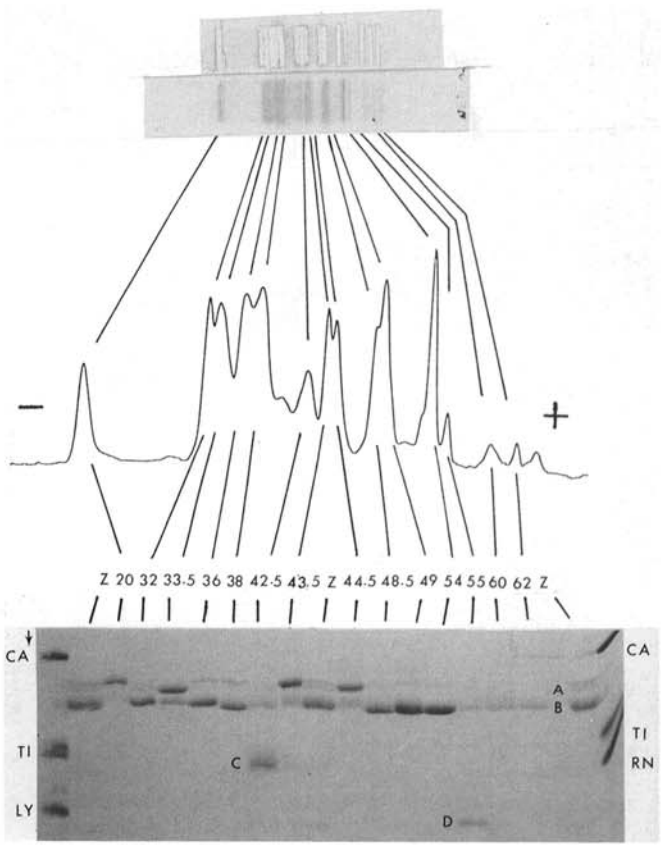

Fig. 4. Isoelectric focusing (IEF) of zein from the inbred N28, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of individual bands. Stained zein bands were removed from the upper IEF gel, while the second IEF gel was scanned (see Fig. 1 for numbering). IEF bands were transferred to SDS-PAGE sample slots as described in the text. A-, B-, C-, and D-zeins are identified in Figure 3. **Z**, purified total zein; **CA**, carbonic anhydrase, 30 kDa; **TI**, soybean trypsin inhibitor, 21 kDa; **LY**, lysozyme, 14.3 kDa; and **RN**, ribonuclease A, 13.7 kDa.

reproducible than bands of A- and B-zein. Bands 60 and 62 from N28 were too faint to be marked on the standard IEF map (Fig. 1). Band 60 is a major band for WF9 and W64A, and was found to be an A-zein (Wilson, *unpublished*); the minor band 60 from N28 was found to be a B-zein (Fig. 4). Thus, it may not be unusual for an IEF band to reveal different sizes in different inbreds. It will be necessary to check each band of each inbred to fully confirm identity. This heterogeneity adds to the apparent number of different zein polypeptides in any inbred. Work is in progress to classify the common zein polypeptides of common inbreds by IEF position and SDS-PAGE size class.

Protein Staining

The assays reported here are made easier by the high sensitivity of the Coomassie Blue R staining systems. As reported earlier (Wilson 1984), the agarose-IEF patterns require a fifth to a tenth as much zein as that needed for PAG-IEF systems (Valentini et al 1979, Wall et al 1984), probably because the agarose gel is very thin when stained. A number of staining and destaining regimes were tested after SDS-PAGE. Rapid staining could be performed with an alcoholic solution without affecting the staining of any proteins. However, all destaining solutions which contained alcohol, such as the common Weber and Osborn (1969) mixture (acetic acid, methanol, and water, 75:50:875), reduced the intensity of staining of zein and of some molecular mass standards. Destaining gels with 12½% TCA required more time, but the zein peak heights measured with a gel scanner were 2–2.5 times higher than when other destaining solvents were used. Good banding patterns were produced in SDS-PAGE gels with 12 μg of total zein (Z in Fig. 4).

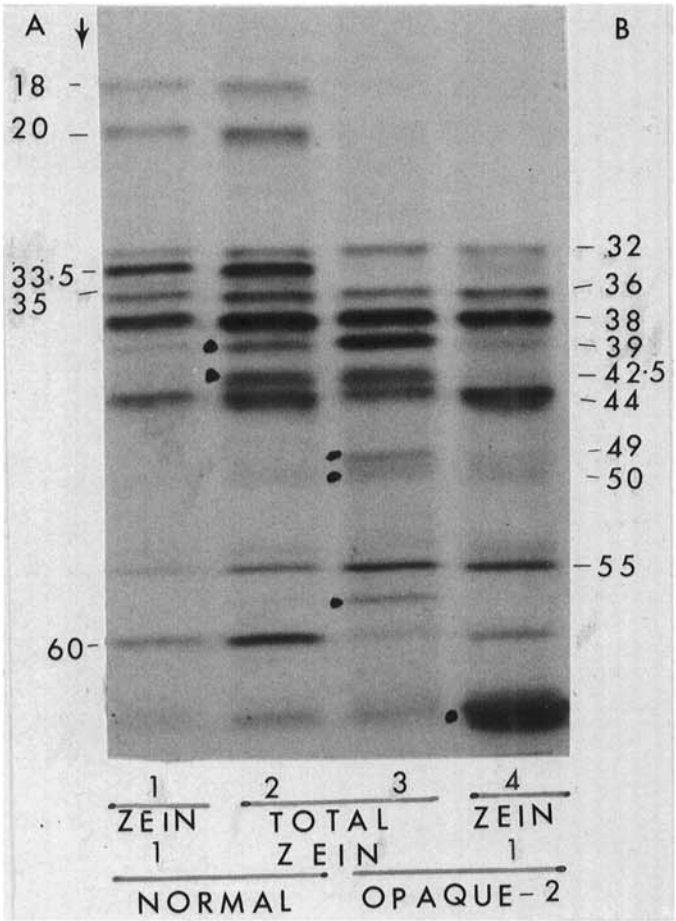

Fig. 5. Effects of the *opaque*-2 mutation and of mercaptoethanol (2-ME) on isoelectric focusing (IEF) band patterns of zeins from the inbred W64A. IEF bands noted on the left side were reduced in the mutant. Bands marked with a dot were extracted only when 2-ME was present in the alcoholic solvent of a total zein extract.

Apparent Molecular Mass and a Zein Nomenclature

The apparent molecular masses of zeins determined using standard proteins (Fig. 4) were: A-zein, 26.5; B-zein, 24; C-zein, 18; and D-zein, 13.5 kDa. These numbers are closer to the values calculated from cDNA sequencing data (Hu et al 1982) for A- and B-zeins than to the results usually reported for SDS-PAGE (Gorinstein et al 1983), but no good explanation is apparent. In Figure 4 A- and B-zeins fall midway between carbonic anhydrase and soybean trypsin inhibitor. Dierks-Ventling (1982) used the same standards and the same gel system, but with 6*M* urea in the gel. A-zein then ran almost as fast as trypsin inhibitor to give a 22 kDa value. Nielsen and Reynolds (1978) suggest that "the use of urea in SDS polyacrylamide gel electrophoresis is unsound" when the object is to determine the molecular weight. Unpublished results (K. Pedersen, reported by Larkins 1983) on the amino acid composition of C-zein derived from cDNA clone sequencing give an estimated mass slightly over 18 kDa. Contrasting results such as these may cause confusion if SDS-PAGE zein bands are identified by apparent molecular masses, for the numbers 21,000–24,000 could refer to either the larger or the smaller of the two major zein bands. The A, B, C, and D nomenclature suggested here is neutral concerning apparent size but does identify the order in which the zeins run on SDS-PAGE. This can provide a simple cross reference because the same zein polypeptide might be reported as "A22" from SDS-PAGE and as "A27" from cDNA data.

A nomenclature system may be developed using the identifications reported here using three terms: SDS-PAGE size class with apparent molecular mass, kDa/IEF band number/inbred. For example, the first three IEF bands in Figure 4 would be identified as A27/20/N28, B24/32/N28, and A26/33.5/N28. Further work is needed to determine if there is a B24/33.5/N28—it could be a contaminant from the adjacent IEF band, or the 33.5 IEF band may consist of two zeins with different sizes. Additional terms could be added as more information is obtained from other assays, such as acid-urea PAGE (Wall et al 1984, Izquierdo et al 1984), HPLC (Bietz 1983), or gene location (Valentini et al 1979, Hastings et al 1984).

A very similar nomenclature, developed using IEF in PAG, has been in use for several years (Soave and Salamini 1984). The most recent revision was reported by Hastings et al (1984). The agarose-IEF system can be recommended because it is more convenient, it gives better separations of zeins, and a calibration system has been developed which allows comparisons among laboratories (Fig. 1 and Wilson 1984, 1985). Further, the nomenclature of Soave and Salamini (1984) makes no provision for the addition of new IEF bands. It is hoped that cross references between the two systems can be made, for a large amount of data has been accumulated using the PAG system.

Variations Caused by Mutation and a Reducing Agent

Figure 5 illustrates the detail obtained by IEF in agarose for the effects of the *opaque*-2 mutation and of 2-ME on zein polypeptide patterns. The bands identified on the left side of the figure are those eliminated or reduced by the mutation. Bands marked with a dot are extracted only in the presence of 2-ME. Band 42.5 has been identified as a C-zein (Fig. 4), but other identities have not yet been determined for this inbred. It seems that the relative amounts of most zein polypeptides are not affected by the presence or absence of 2-ME, while others are found only when 2-ME is used.

The systems reported here should prove useful for identifying inbreds, for determining seed purity, for genetic studies on chromosome locations of zein genes, for determining identities of zein polypeptides synthesized in vitro, and for assessing the extractability and solubility properties of different zein polypeptides.

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