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TWO-DIMENSIONAL GEL ELECTROPHORESIS OF ZEIN PROTEINS FROM NORMAL AND OPAQUE-2 MAIZE WITH NON-IONIC DETERGENT ACID UREA-POLYACRYLAMIDE GEL ELECTROPHORESIS IN THE FIRST DIMENSION

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SUMMARY

Different fractions of zeins, the alcohol soluble proteins of maize, were analyzed by non-ionic detergent acid urea-polyacrylamide gel electrophoresis (NDAU-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). By using NDAU-PAGE, the zein-1 fraction can be resolved at least in 7 polypeptide components, while only 3 protein bands can be observed by SDS-PAGE. Zein-2 showed at least 2 additional components as compared with zein-1 when analyzed by SDS-PAGE and a total of 10 bands in the presence of non-ionic detergent, acetic acid and urea. Combining both techniques in a two-dimensional arrangement, 16 components can be resolved for zein-1 proteins. This method has been used here to distinguish the protein patterns of a normal variety and one containing the opaque-2 (o2) mutation.

Key words: Non-ionic detergent electrophoresis — Opaque-2 — Two-dimensional electrophoresis — *Zea mays* L. — Zein

INTRODUCTION

Zeins are a class of proteins accounting for 50% of the total endosperm protein at maturity in normal maize varieties. It has been shown that

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Abbreviations: IEF, isoelectric focusing; NDAU-PAGE, non-ionic detergent acid urea-polyacrylamide gel electrophoresis; Nonidet P40, ethylphenylpolyethyleneglycol; o2, opaque-2; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Z_1, zein extracted with 70% ethanol; Z_2, zein extracted with 70% ethanol plus 0.6% 2-mercaptoethanol.

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varieties containing the o2 gene, that effects the nutritional quality of maize grains by increasing its lysine content, have a lower proportion of zeins [1]. Zeins can be classified as zein-1 (Z_1) (alcohol soluble proteins) and zein-2 (Z_2) (proteins soluble in alcohol only in the presence of a reducing agent) according to Sodek and Wilson [2]. Z_1 extracted from both normal and o2 mutant maizes can be resolved into 2 or 3 bands in the 19 000—23 000 dalton region by means of SDS-PAGE [3,4]. Z_2 shows two faster bands at 14 000 and 10 000 daltons, respectively, in addition to the same two bands of Z_1 fraction [5]. Zeins can also be analyzed by isoelectric focusing (IEF) giving 10—15 bands in the 5—9 isoelectric point range [6]. However by using SDS-PAGE or IEF, only small differences can be observed between the zein components of different maize varieties or mutants, e.g., between normal and o2.

NDAU-PAGE has been used in order to take advantage of differences other than molecular weight or isoelectric point [7]. In NDAU-PAGE protein mobility depends on size, charge and hydrophobicity [8]. A clear dependence of electrophoretic mobility with the extent of binding of non-ionic detergent has been reported for globin chains and histones [8,9]. In the present paper, the application of NDAU-PAGE to zeins of normal and the o2 maize mutant is reported. The procedure allows us to distinguish a greater number of zein components than SDS-PAGE, and shows a clear distinction between normal and the o2 mutant.

MATERIALS AND METHODS

Plant material

Mature maize seeds of the double hybrid E-10 and the corresponding o2 mutant, the double hybrid E-41, were used in this study. Zeins were extracted by treatment of defatted endosperm flour with 70% ethanol for Z_1 and 70% ethanol plus 0.6% 2-mercaptoethanol for Z_2 [10]. The extracted material was dialyzed against distilled water and lyophilized.

PAGE in one dimension

SDS-electrophoresis. 15% polyacrylamide slab gel (160 mm long and 1.5 mm thick) electrophoresis in SDS was carried out for 14 h at 25 mA essentially as described by Laemmli [11]. Protein samples containing 100—125 μg in 20—50 μl of sample buffer were loaded in the gel wells.

NDAU-electrophoresis. Ethylphenylpolyethyleneglycol, Fluka (Nonidet P40) acid urea-polyacrylamide gel electrophoresis was performed by the method of Zweidler and Cohen [7] and Puigdomènech and Ruiz Carrillo [12] with some modifications. The separating gel (110 × 240 × 1.5 mm) was composed of 12% acrylamide, 0.1% *N,N'*-methylenebisacrylamide, 0.5% *N,N,N',N'*-tetramethylethylenedaimine, 0.45% Nonidet P40, 8 M urea, 5% acetic acid and 0.08% ammonium peroxodisulfate. The solution containing urea, acrylamide and Nonidet P40 was treated with a Mixed

Bed Resin AG501-XB (D) (Bio-Rad), acetic acid was added afterwards, and the solution was degased. An upper gel was laid containing 7% acrylamide, 0.5% bisacrylamide, and the same components as the separating gel. Running buffer was 5% acetic acid.

A pre-electrophoresis was carried out at 230 V with a layer of 8 M urea, 0.45% Nonidet P40 in 14% acetic acid solution on the top of the gel, until no current decrease was observed. Scavenging of free radicals was carried out by a second pre-electrophoresis after filling the stacking gel wells with a 1 M cysteamine hydrochloride, 7 M urea solution, at 230 V during 60 min. Proteins were dissolved in a 50 mM Tris—HCl (pH 9.0), 8 M urea, 10 mM dithiothreitol buffer solution. After careful cleaning of the gel wells, 20 μl of each sample (100—125 μg of protein) were loaded in the gel and the electrophoresis was run for 20 h at 230 V. The gels were stained with 0.2% Coomassie brilliant blue R (Sigma Co.) for 1 h in 50% trichloroacetic acid and destained with 7% acetic acid (twice) and 7% acetic acid/35% methanol (twice).

The gels were photographed with a Polaroid MP-4 camera.

PAGE in two dimensions

Separation in the first dimension was performed by NDAU-PAGE as described above, except that samples contained 25 μg protein in 15—18 μl of sample buffer. The strips (1 cm wide) of NDAU-PAGE gels were equilibrated with 0.125 M Tris—HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS and 5% 2-mercarptoethanol by two sequential 15-min incubations at room temperature.

Separation in the second dimension was then performed by SDS-electrophoresis as described above. A NDAU-electrophoresis gel strip was introduced on the top of the stacking gel avoiding the formation of air bubbles and the upper reservoir buffer was poured. Electrophoresis was run for 14 h at 25 mA, and the gel stained with silver, essentially as described by Morrissey [13].

RESULTS AND DISCUSSION

One dimensional SDS-PAGE of zeins

Zeins, the corn prolamines (alcohol soluble proteins of cereals) reveal a relative homogeneity in size when analyzed by SDS-electrophoresis [4,14]. In long 15% polyacrylamide gels, the Z_1 fraction from normal maize of E-10 variety is resolved in 3 bands (Fig. 1a). The slowest bands (M_r 21 500 and 21 000) form a doublet and the fastest component (M_r 19 000) shows higher intensity than the other bands. The Z_2 fraction contains two additional components of mol. wts. 13 500 and 10 000. (Fig. 1c). It is generally considered that the 19 000 dalton band corresponds to the main zein component [3—5]. However, some authors found only 1 band in the region of 23 000—21 000 daltons [4,14] or at 21 800 daltons [3,5]. The molecular

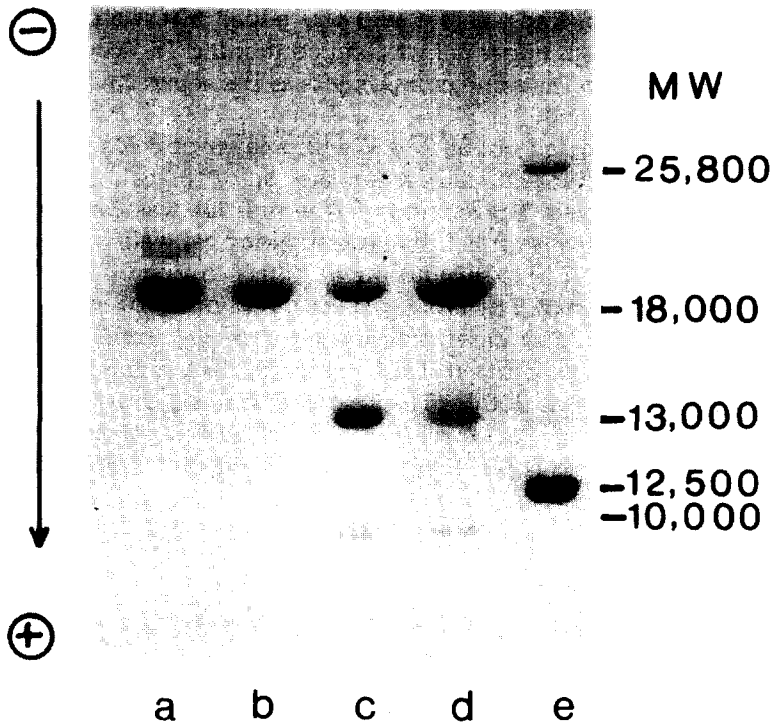

Fig. 1. SDS-PAGE of zeins. (a) 100 μg Z_1 normal maize. (b) 100 μg Z_1 o2 maize. (c) 125 μg Z_2 normal maize. (d) 125 μg Z_2 o2 maize. (e) Molecular weight markers (15 μg total protein) are concanavalin A polypeptides: 25 800—18 000—13 000—10 000, respectively, and cytochrome *c* 12 300 daltons.

weights of protein bands were estimated following Shapiro et al. [15], using as protein standards, the polypeptides of concanavalin A from *Canavalia ensiformis* and cytochrome *c* (Fig. 1e). The molecular weight values for the different zein polypeptides so obtained, are similar to the values calculated by other authors [16].

Z_1 and Z_2 from o2 maize shows similar electrophoretic patterns to Z_1 and Z_2 from normal maize. The intensity of the 21 500 and 21 000 dalton bands decreases relative to 19 000 dalton bands of normal and a faint band of 14 000 daltons appears in Z_2 from o2 (Fig. 1b and 1d). This result is in agreement with the effect of o2 described by other authors [3,17].

NDAU-PAGE of zeins

In contrast to SDS-PAGE the heterogeneity of zeins is clearly shown by NDAU-PAGE (Fig. 2). Between seven and thirteen polypeptide components can be resolved depending on the sample studied. Z_1 fraction from normal maize shows seven components (Fig. 2a). Normal Z_2 can be

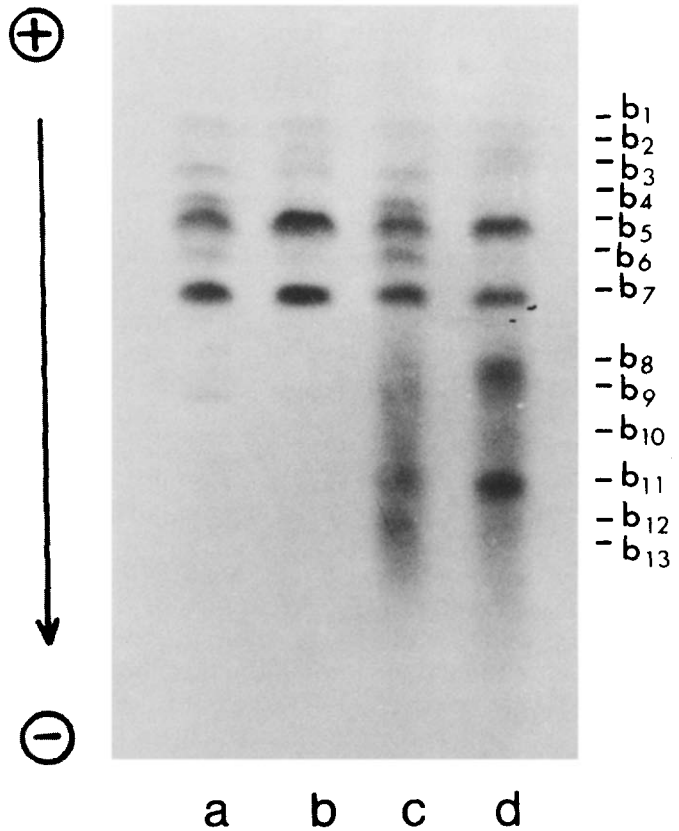

Fig. 2. NDAU-PAGE of zeins. (a) 100 μg Z_1 normal maize. (b) 100 μg Z_1 o2 maize. (c) 125 μg Z_2 normal maize. (d) 125 μg Z_2 o2 maize. The gel was stained with Coomassie blue.

easily distinguished from Z_1 (Fig. 2c) by the observation of 5 new bands (b8, b10—13).

NDAU-PAGE provides a method to distinguish clearly the zein fractions of normal and o2 maize. In Z_1 from o2 a new band (b2) appears, another disappears (b4) and the intensity of b6 is decreased as compared with Z_1 from normal maize (Fig. 2b). The protein band patterns of Z_2 present similar differences between normal and o2, though the situation is more complicated, especially in the high mobility region. One component appears (b2), two components disappear (b4, b6) and other components change their intensity (Fig. 2d), or present a more diffuse aspect (b9, b12, b13).

Two-dimensional electrophoresis of zeins

Two-dimensional electrophoresis using NDAU-PAGE in the first dimension and SDS-electrophoresis in the second, allows on one hand, the identification of the bands observed in the first technique in terms of their mobil-

ity in SDS-PAGE, the most commonly used methodology for examination of zeins. On the other hand, it provides an alternative to the O'Farrell method [18] to resolve a high number of zein polypeptides. The patterns obtained from a two-dimensional analysis of the zein-1 fraction from normal and o2 maizes are shown in Figs. 3 and 4.

At least 16 protein components can be shown for the zein-1 fraction of normal maize distributed between three classes of molecular weight: 5, 2 and 6 components for the molecular weight classes of 21 500, 21 000 and 19 000 daltons, respectively (Fig. 3). The pattern obtained from a two-dimensional electrophoresis of Z_1 o2 fraction is similar to the normal Z_1 showing 16 polypeptide components (Fig. 4). However, two spots of the 21 500 dalton class and another one of the 21 000 dalton class are missing in relation to the normal Z_1. Three new spots in the 19 000 mol. wt. range appear in the o2 gel, probably due to the gain in intensity of the 19 000 dalton class relative to the decreased 21 500 and 21 000 dalton bands in the o2 Z_1. The arrows in each figure show the spots that do not appear in the other one.

As described previously, zeins are quantitatively decreased by the o2 mutation [1] The reduction caused by o2 in E-10 is from 60% to 25% of total endosperm proteins. In agreement with other authors [19,20], the results reported here show a relative reduction by o2 in the high molecular weight (21 500 and 21 000) classes of zeins. Besides, these results show an

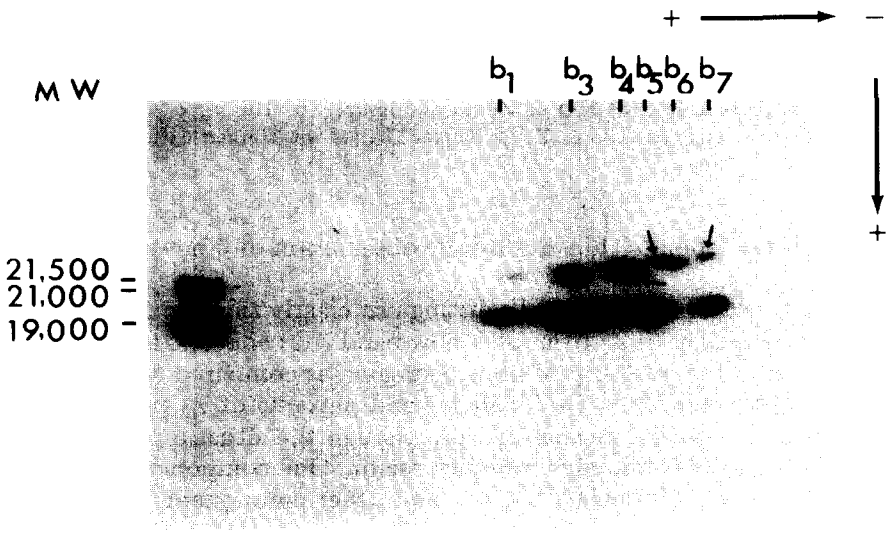

Fig. 3. Two-dimensional electrophoresis of normal zein-1. Normal Z_1 (25 μg) was run in a 12% NDAU-polyacrylamide slab gel (migration was from left to right). The lane was excised, equilibated and run in a 15% SDS-polyacrylamide slab gel (migration was from top to botton) and stained with silver stain. Normal Z_1 (2 μg) was loaded on a well at the left side of the gel. The arrows indicate the spots that show a larger change in intensity when compared with the same samples of an o2 mutant (see Fig. 4).

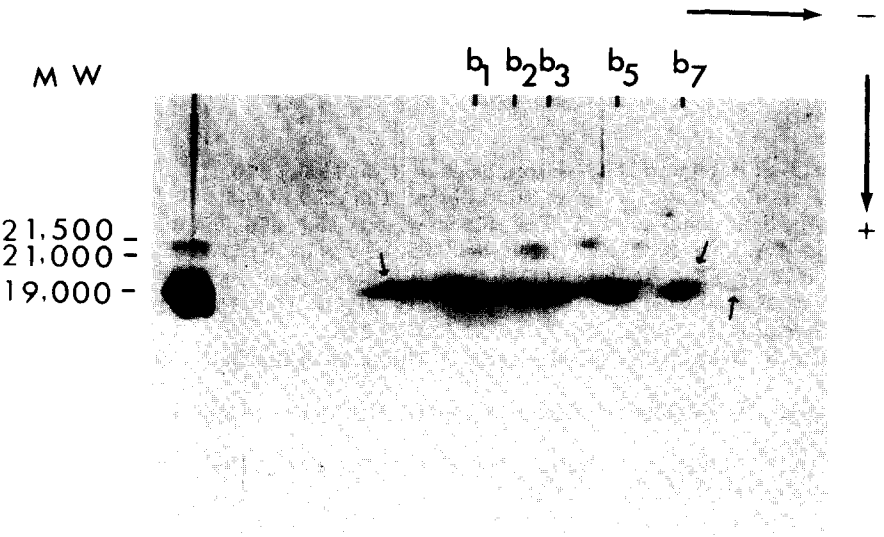

Fig. 4. Two-dimensional electrophoresis of o2 zein-1. o2 Z_1 (25 μg) was run in a 12% NDAU-polyacrylamide slab gel (migration was from left to right). The lane was excised, equilibrated and run in a 15% SDS-polyacrylamide slab gel (migration was from top to bottom). 2 μg of o2 Z_1 were loaded on a well at the left side of the gel.

increase in the number and intensity of bands from 19 000 dalton class when equal amounts of total protein are examined.

Several precautions have been taken in order to avoid effects on mobility other than those coming from the microheterogeneity of the zein polypeptides. The possible carbamylation of proteins by cyanate from urea has been avoided, using fresh preparations of protein and pretreating the urea solution with a mixed bed ion-exchanger. Methionine oxidation does not take part because oxidants have been scavenged from the gels and zeins-1 have a very low content in methionine. Furthermore, patterns are reproducible run to run, and sample to sample.

The present report shows that NDAU-PAGE is an alternative electrophoretic method to SDS-PAGE and IEF that allow a clearer differentiation between wild-type and o2 showing high resolution of zein polypeptides, both with Z_1 and Z_2. A combination with the SDS-PAGE in a two dimensional system allows to size the bands observed in the first dimension NDAU-PAGE. Furthermore, a larger number of bands appears in this system thus permitting the resolution of zeins-1 into 16 different polypeptides.

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