Differences in Corn Endosperm Proteins in Developing Seeds of Normal and *Opaque-2* Corn

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ABSTRACT

The effects of the *opaque-2* (*o2*) mutation on the composition of corn endosperm proteins and their relative rates of syntheses during seed development were investigated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and two-dimensional electrophoresis. Nonprotein nitrogen, albumins and globulins, zeins, alcohol-soluble reduced glutelins (ASG), and alcohol-insoluble reduced glutelins (AIG) were sequentially extracted from ground endosperm dissected from corn kernels harvested at 18, 22, 30, and 48 days after pollination. In early stages of normal genotype, nonprotein nitrogen, albumins, and globulins were present at high levels. These components declined in amount, and levels of zein, ASG, and AIG were elevated during maturation. In the *o2* grain, the level of zein did not rise as high, and the final amount of AIG was higher than that of normal endosperm. Compositions of albumin and globulin proteins, as shown by SDS-PAGE, were similar at the same stages of grain development in normal and *o2* corns, except that albumins of normal endosperm contained, at all stages, a protein absent in *o2* albumins. Zein, upon SDS-PAGE, showed less of the higher molecular weight proteins in the *o2* genotype. Two-dimensional electrophoresis (isoelectric focusing in the first direction, pH 3.5 aluminum lactate PAGE in the second) showed that *o2* corn lacked several zeins present in normal and other mutant genotypes. SDS-PAGE indicated that certain albumins and globulins diminished during maturation of both normal and *o2* kernels. ASG, like zein, did not change in SDS-PAGE pattern during maturation. AIG patterns of certain components became more intense during kernel development, indicating that these proteins are synthesized mainly during maturation. These changes were discussed with respect to effects of the *o2* gene on kernel composition and structure.

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The discovery by Mertz et al (1964) that a mutant gene *opaque-2* (*o2*) in corn increases lysine and tryptophan contents of endosperm protein encouraged development of corn grain of better nutritional quality for feed and food to supplant existing hybrids deficient in those amino acids. Improved nutritional quality of *o2* grain results from a change in its endosperm protein composition: it has a reduced level of zein, which is deficient in lysine and tryptophan, and increased amounts of albumin, globulin, and glutelin proteins, which have higher levels of those amino acids (Mertz et al 1964, Mossé et al 1966). Introduction of the mutant *o2* gene into inbred lines used to produce hybrid seed resulted in corn lines that had two major restrictions—lower yield and soft, friable kernels. However, by introducing modifier genes into *o2* lines, Vasal et al (1984) found that corn with better grain yield and kernel structure may be obtained.

Two-dimensional electrophoresis reveals that zein consists of many closely related polypeptides; a single inbred may have 25 to 40 different zeins (Hagen and Rubenstein 1980, Wall et al 1984), and the haploid maize genome may have more than 100 zein genes (Viotti et al 1979). Genes for zein syntheses have been isolated, first by formation of cDNA from mRNAs that are translated to produce zeins, and also from genomic DNA (Geraghty et al 1981, Pederson et al 1982, Kridl et al 1984). Zein has been synthesized in vitro by using mRNA produced from the zein genes. These studies establish that each zein is coded by a single gene or duplicate genes. The zein genes have been located in clusters on adjacent regions of chromosomes 4, 7, and 10 (Viotti et al 1982). The *o2* gene appears to mainly diminish production of higher molecular weight zeins, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Lee et al 1976). Whereas the *o2* gene is located on chromosome 7, these higher molecular weight zeins appear to be coded primarily by genes on chromosome 4. Amino acid sequences of zeins were first compared by amino-terminal sequence analyses (Bietz et al 1979) and were fully established by sequencing their cDNAs and genomic DNAs (Geraghty et al 1981, Pederson et al 1982, Kridl et al 1984). Zeins have similar C- and N-terminal regions and a central structure of eight or nine tandem repeating units of about 20 amino acids; light and heavy chains differ mainly in number of repeat units (Spena et al 1982).

Although considerable information is now available concerning syntheses of corn endosperm proteins, it is not known how the *o2* mutation induces the numerous changes in kernel structure and protein composition. The normal allele at the *O2* locus evidently produces a regulatory protein that induces expression of other genes, including some for zein production. Soave et al (1981) found that the *o2* mutation also curtails production of 32 kilodalton

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(kDa) salt-soluble proteins that exist as two species differing in isoelectric point (B_{32}-Ac and B_{32}-Bas). The B_{32} proteins do not appear to be directly coded by the normal *O2* gene, but by the normal allele *O6*, which requires the normal *O2* gene for expression. The *o6* gene in a homozygous inbred is lethal. The *o2* gene also redistributes endosperm proteins compared to normal dent-type kernels. According to Wolf et al (1969), the dent kernel has a peripheral vitreous portion that contains small cells packed with protein bodies composed of zein. In the opaque kernel, protein is distributed more uniformly through the endosperm, which has more uniform cells with fewer and smaller protein bodies and has air pockets between cells. Christianson et al (1974) noted increased production of glutelin-containing particulates and fewer and smaller zein-rich protein bodies in early stages of grain development in *opaque-2* corn.

We have now sought to establish whether any additional significant differences occur in albumins, globulins, zeins, and glutelins of normal and *o2* corn endosperm by means of SDS-PAGE. By following changes in these proteins in endosperms of different maturity, we sought to establish whether the mutation delays or inhibits synthesis of specific proteins, and to establish when maximum production of those proteins occurs. This information may provide a basis for understanding how complex compositional and structural changes are induced by the *o2* mutation in corn and how the normal *O2* gene regulates these diverse phenomena.

MATERIALS AND METHODS

Corn Extract Preparations

Normal W64A inbred corn and high-lysine *o2*, *fl2*, and *su2* mutants were provided by David Glover, Purdue University, West Lafayette, IN. As a result of extensive backcrossing with normal W64A, these mutant inbreds were near-isogenic except for the mutant genes. They were grown in a field plot in 1984 at the Northern Regional Research Center, Peoria, IL. Plants were self-pollinated and harvested at 18, 22, 30, and 48 days after pollination; grains were stored at −22°C. Endosperms were dissected by hand, weighed, lyophilized, reweighed, and ground to −40 mesh in a Udy cyclone mill in the presence of dry ice.

Ground corn samples (5.0 g) were extracted twice at 4°C (1 hr each time) with 50 ml of cold 0.5*N* NaCl by stirring with a magnetic stirrer. After centrifugation of each extract (15,000 rpm [25,400 × *g*], Beckman L8-70 M ultracentrifuge), supernatants were combined. To separate proteins from nonprotein nitrogen (NPN), an aliquot of the total supernatant was mixed with an equal volume of 20% trichloroacetic acid (TCA) and centrifuged at 15,000 rpm. The supernatant was extracted with an equal volume of diethyl ether to remove TCA. The aqueous phase was retained for analysis of NPN. The precipitate was dispersed in water for determination of total albumin and globulin protein. The remaining 0.5*N* NaCl extract of salt-soluble proteins was dialyzed against 8 L of water (four changes) for 48 hour at 4°C. The dialyzed suspension was centrifuged; the precipitate was primarily globulins, and the supernatant contained albumins. These were each lyophilized.

Residues from the endosperm meal were then extracted twice at room temperature by stirring for 2 hr with 100 ml of 70% ethanol + 0.5% sodium acetate to yield zein. After centrifugation, supernatants were combined. Residues were next extracted for 2 hr with 50 ml of 70% ethanol + 0.5% sodium acetate + 2% 2-mercaptoethanol (2-ME), twice at room temperature, to obtain alcohol-soluble glutelins (ASG). After centrifugation, these ASG extracts were combined. Residues were finally extracted twice (2 hr at room temperature) with 50 ml of pH 8.2 borate buffer containing 2% 2-ME; extracts were combined after centrifugation to obtain alcohol-insoluble reduced glutelin (AIG). Nitrogen was determined on aliquots of all extracts by a micro Kjeldahl procedure to calculate protein yields; percent protein was expressed (recognizing the probable error in the protein/N conversion factor) as 6.25 × %N. Remainders of each extract were dialyzed against 8 L of water (four changes) over 48 hr at 4°C and lyophilized.

Electrophoresis

SDS-PAGE was conducted in a vertical Hoefer SE600 apparatus on 1.5-mm gels. Running gels were made with either 10 or 14% total monomer (acrylamide and bisacrylamide), of which 2.7% was bisacrylamide, in pH 8.8 0.375*M* Tris-Cl buffer containing 0.1% SDS; the stacking gel was pH 6.8 0.125*M* Tris buffer containing 0.1% SDS and 4% total monomer (of which 2.7% was bisacrylamide). Gels were polymerized with tetramethylenediamine and ammonium persulfate. Proteins (2 mg) were dissolved in 0.5 ml of treatment buffer (0.63*M* Tris-Cl, pH 6.8, containing 2% SDS, 10% glycerol, and 5% 2-ME) and heated 90 sec at 100°C; 20 μl was applied to each well. Standard reference proteins from Bio-Rad (Richmond, CA) of low molecular weight (lysozyme [14.4 kDa], soybean trypsin inhibitor [21.5 kDa], carbonic anhydrase [31.0 kDa], ovalbumin [45.0 kDa], bovine serum albumin [66.2 kDa], and phosphorylase B [92.5 kDa]) and high molecular weight (ovalbumin [45.0 kDa], bovine serum albumin [66.2 kDa], phosphorylase B [92.5 kDa], β-galactosidase [116.25 kDa], and myosin [200.0 kDa]) were introduced into adjacent wells. The tank buffer was 0.025*M* Tris, pH 8.3, containing 0.0192*M* glycine and 0.1% SDS. Electrophoresis was conducted at 30 mA per gel until marker dyes were 1 cm from the bottom of the gel (approximately 3 hr). Gels were stained with a solution of 40 g of TCA, 120 ml of methanol, and 240 ml of water and destained with a mixture of 200 ml of methanol, 40 ml of acetic acid, and 160 ml of water.

Two-dimensional electrophoresis was conducted by the method of Wall and co-workers (1984), by using isoelectric focusing (IEF) in polyacrylamide gels in the first dimension and pH 3.5 aluminum lactate polyacrylamide gel electrophoresis in the second. Both separations were conducted on an LKB Multiphor (model 2117) horizontal unit with 27 × 11.5 × 0.2 cm slab gels (5% total monomer; 0.127% bisacrylamide).

For IEF, gels contained 6*M* urea and 2% LKB pH 6–8 ampholines. Proteins (6 mg in 100 ml of 6*M* urea) were reduced and alkylated as described by Paulis and Wall (1977). Samples were absorbed on 1.0 × 0.5 cm Cambrele wicks. The positive electrode strip was soaked in saturated aspartic acid, and the negative in 0.1*M* lysine. IEF was conducted at 4°C at 13 W constant power for 4 hr. Gel strips for the second dimension were sectioned from the center of IEF lanes with a double-bladed knife with edges 2 mm apart.

The second dimension separation was as described by Wall et al (1984). Briefly, the aluminum lactate gel was prepared in a holder with two inserts for wells for the reference samples. The gel consisted of 2.53 g of acrylamide, 0.154 g of bisacrylamide, 28.83 g of urea, 0.0295 g of aluminum lactate, 0.25 ml of lactic acid, and *N,N,N′,N′*-tetramethylethylenediamine (225 μl), made to 60 ml with deionized water. After degassing, 1.3 ml of 1.0% ammonium persulfate was added, and the gel solution was introduced into the gel holder. Troughs at the ends of the electrophoresis apparatus were filled with aluminum lactate buffer (0.884 g of aluminum lactate, 3.6 ml of lactic acid, and 432.4 g of urea diluted to 1,800 ml); electrode wicks bridged gel and tank buffer solution. The gel was pre-electrophoresed 6 hr at 13 W constant power at 4°C. Following pre-electrophoresis, two slots were cut in the gel with the double-bladed knife as described by Wall et al (1984), and the first dimensional gel strips were inserted with proper orientation so that two separations were performed on each gel. Electrophoresis was performed at 4°C and 7.8 W constant power for 16 hr. Silver staining of the gels was done according to the procedure of Merrill et al (1981).

RESULTS

Yields of Proteins

Figure 1 compares changes in protein composition of normal and *o2* W64A endosperms during kernel developnent. In agreement with Murphy and Dalby (1971), 0.5*N* NaCl-soluble nitrogen compounds constituted a major class of nitrogen-containing components in 18-day normal seed, exceeded only by zein. Albumins and globulins together accounted for a greater percentage of extracted nitrogen than did NPN at all stages of

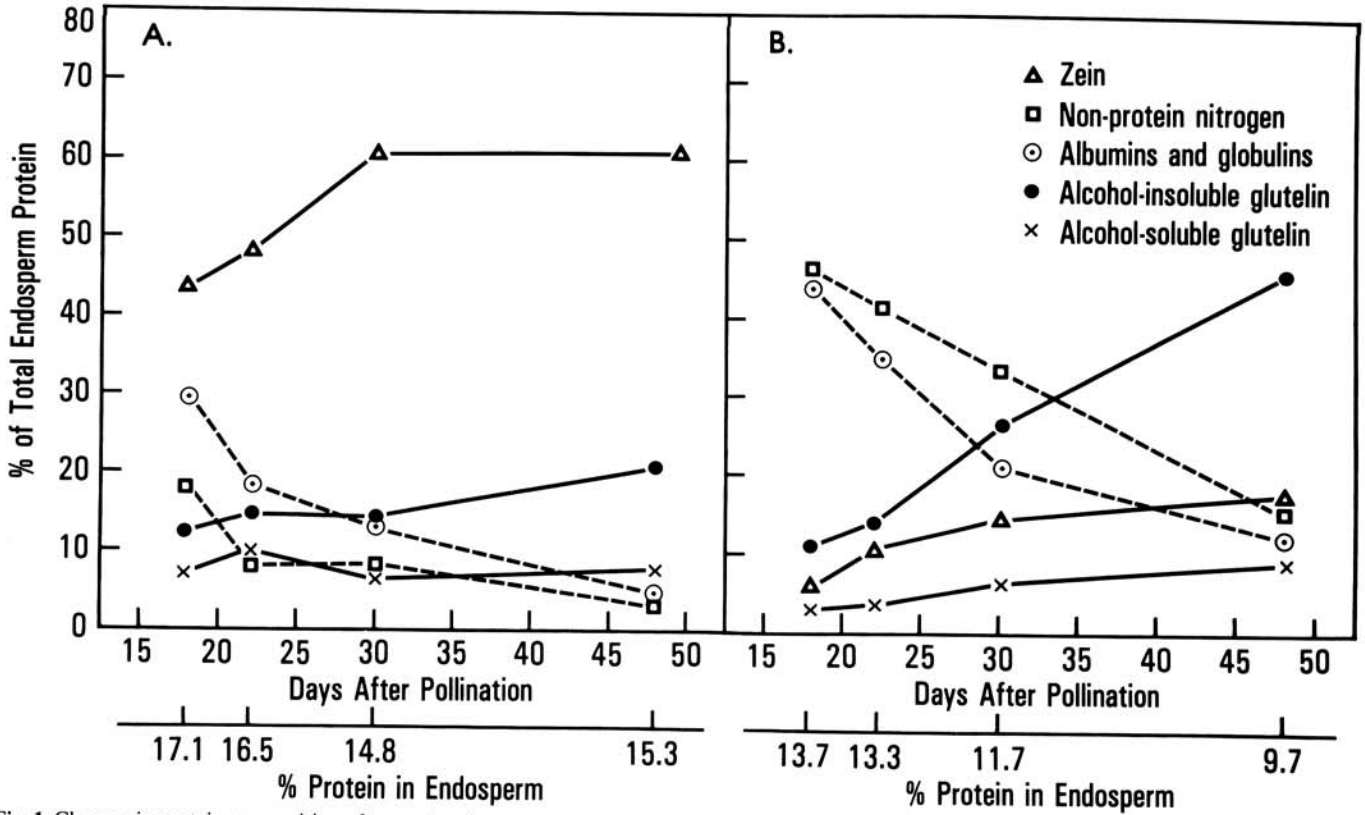

Fig. 1. Changes in protein composition of normal and *opaque-2* W64A corn endosperm during grain development expressed as percent of total protein. **A,** W64A normal; **B,** W64A *opaque-2*.

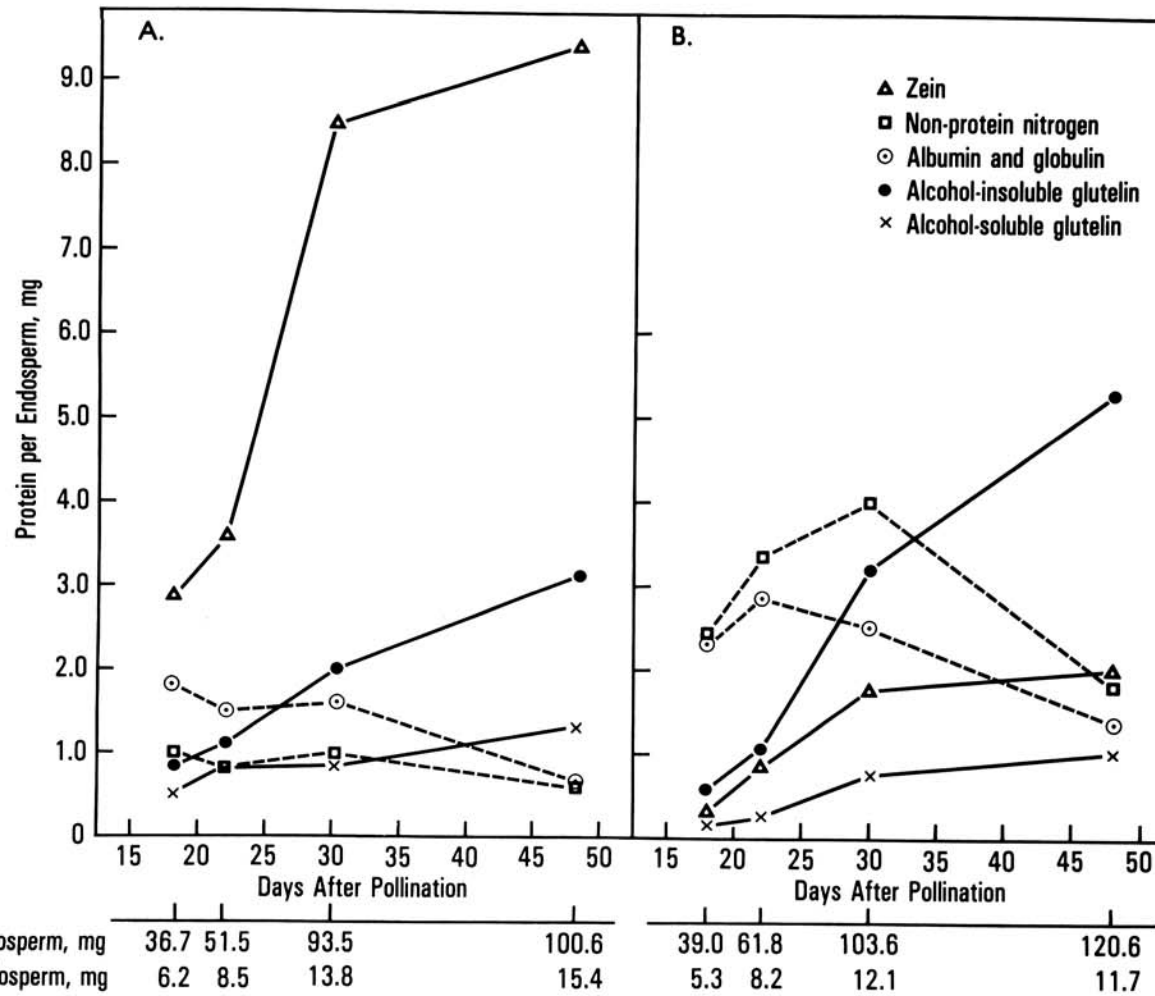

Fig. 2. Changes in amounts of different protein fractions in normal and *opaque-2* corn endosperm during grain development expressed on a milligrams per endosperm basis. **A,** W64A normal; **B,** W64A *opaque-2*.

development. The proportion of the total nitrogen extracted with saline solution (NPN plus albumins and globulins) from *opaque-2* grain was twice that of the normal endosperm. However, NPN exceeded albumin plus globulin nitrogen content at all stages of development of *o2*. In both corn genotypes, the percentages of albumins, globulins, and NPN decline with maturity; however, the degree of decline was greater in normal than in *o2* endosperm. The level of albumins and globulins remained much higher in 48-day *o2* than in normal. The high NPN content of the *o2* endosperm consists mainly of amino acids (Misra et al 1975). The quantity of zein increased rapidly in normal corn, constituting about 60% of the total protein by 48 days. In contrast, in the *o2* endosperm the rate of zein increase was much less, and zein constituted only 20% of the total protein at 48 days. The fraction of total protein extracted by 70% ethanol + 0.5% sodium acetate did not increase greatly in either normal or *o2* endosperms during kernel development. The proportion of alcohol-insoluble glutelin fraction increased only slightly in normal endosperm, but was elevated considerably in *o2*, paralleling the decline in albumins and globulins. About 5% of the protein was not extracted in most of the samples.

When these data were expressed as milligrams of protein per endosperm (Fig. 2), similar conclusions resulted, supporting earlier observations of curtailed zein production in the *o2* mutant, along with increased amounts of NPN, albumins, globulins, and especially AIG.

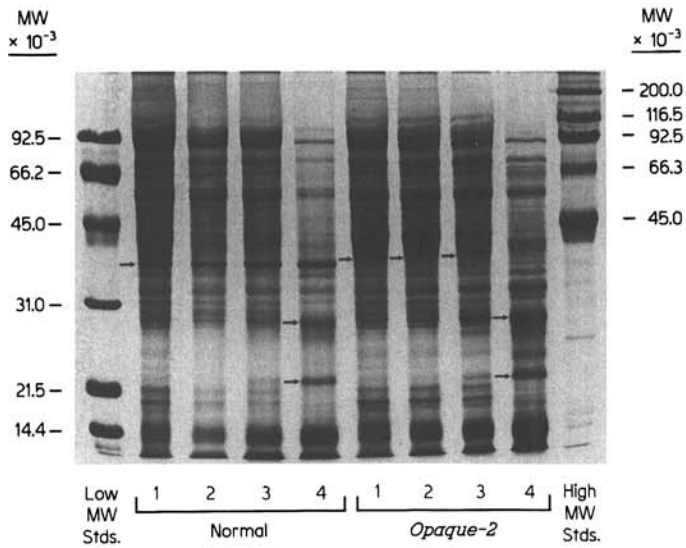

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of albumins from normal and W64A *opaque-2* endosperms at **1,** 18 days; **2,** 22 days; **3,** 30 days; and **4,** 48 days after pollination. Arrows indicate components referred to in text.

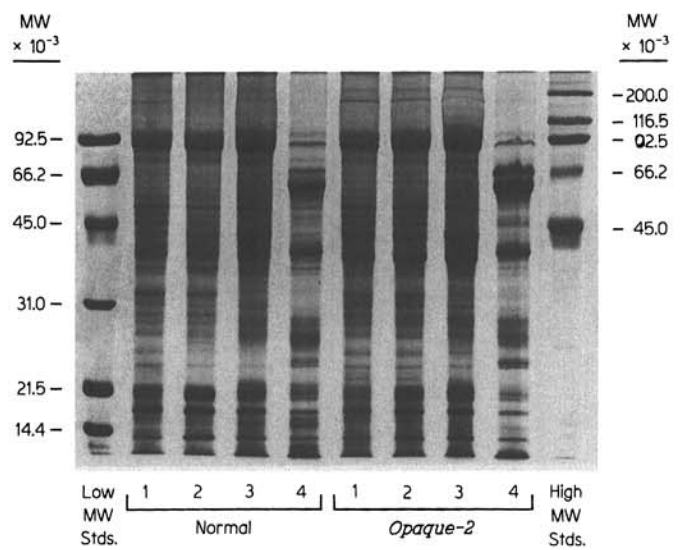

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of globulins from normal and W64A *opaque-2* endosperms at **1,** 18 days; **2,** 22 days; **3,** 30 days; and **4,** 48 days after pollination.

SDS-PAGE of Corn Endosperm Proteins

Figure 3 illustrates SDS-PAGE patterns of albumins extracted from normal and *o2* endosperms at 18, 22, 30, and 48 days past pollination. One marked difference between normal and *o2* albumins at each stage was a component at about 37,000 mol wt that was present in normal lines but absent in *o2* albumins. (This component, and others referred to in the text are indicated by arrows.) A band at about 39,000 mol wt was more prominent in 18-, 22-, and 30-day *o2* patterns, but was faint in albumin extracts from normal endosperms of the same stage of maturation. Otherwise, albumins of normal and *o2* W64A appear markedly similar qualitatively at each stage of development. In the later stages (especially 48-day), both normal and *o2* albumins showed decreased intensity of many bands. However, bands at 22,000 and 29,000 mol wt were more prominent in the 48-day extracts than in earlier stages in both genotypes.

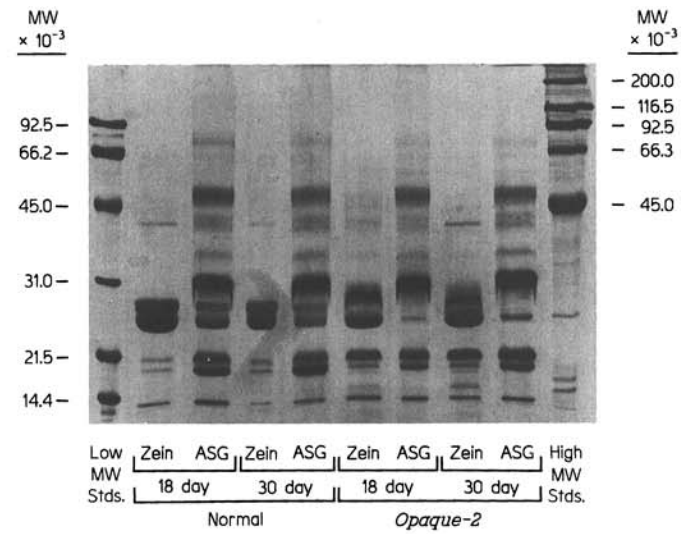

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of zein (70% ethanol + 0.5% sodium acetate) and alcohol-soluble glutelin (**ASG**) (70% ethanol + 0.5% sodium acetate + 2% mercaptoethanol) extracts from normal and *opaque-2* corn endosperm at different stages of development.

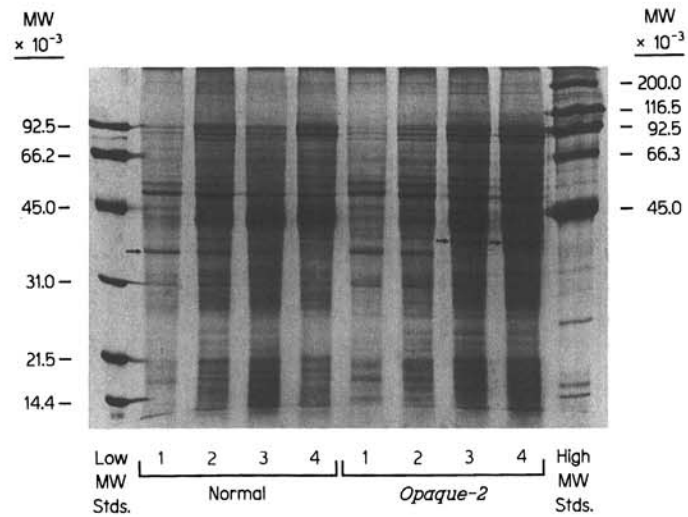

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of extracts of alcohol-insoluble glutelins (pH 8.2 borate buffer containing 0.5% SDS and 2% mercaptoethanol) from normal and *opaque-2* endosperms at **1,** 18 days; **2,** 22 days; **3,** 30 days; and **4,** 48 days after pollination. Arrows indicate components discussed in text.

SDS-PAGE patterns of globulins from developing W64A normal and *o2* endosperms were similar at comparable stages of maturity (Fig. 4). Again, certain bands prominent at earlier stages in both genotypes were less apparent at 48 days, but other bands were intensified. These changes may signify disappearance of certain globular proteins as the content of seed globulins is reduced, whereas residual soluble globulins appear more abundant.

Figure 5 compares zeins and ASGs of normal and *o2* endosperms at 18 and 30 days of grain development. The two major zein bands (24,000 and 26,000) were present in 70% ethanol + 0.5% sodium acetate extracts, along with minor amounts of lower molecular weight polypeptides resembling ASGs. The ASGs have polypeptides with apparent molecular weights of 12,000, 16,000, 19,000, 28,000, and 48,000, as well as the two zein bands, probably caused by disulfide crosslinks between some zein chains. There appeared to be more ASG contaminant in the 18-day zein extract and more zein contaminant in the 30-day ASG extract; thus, disulfide bond formation appeared to progress with time. Zein extracts of *o2* endosperm had less 26,000 mol wt protein and

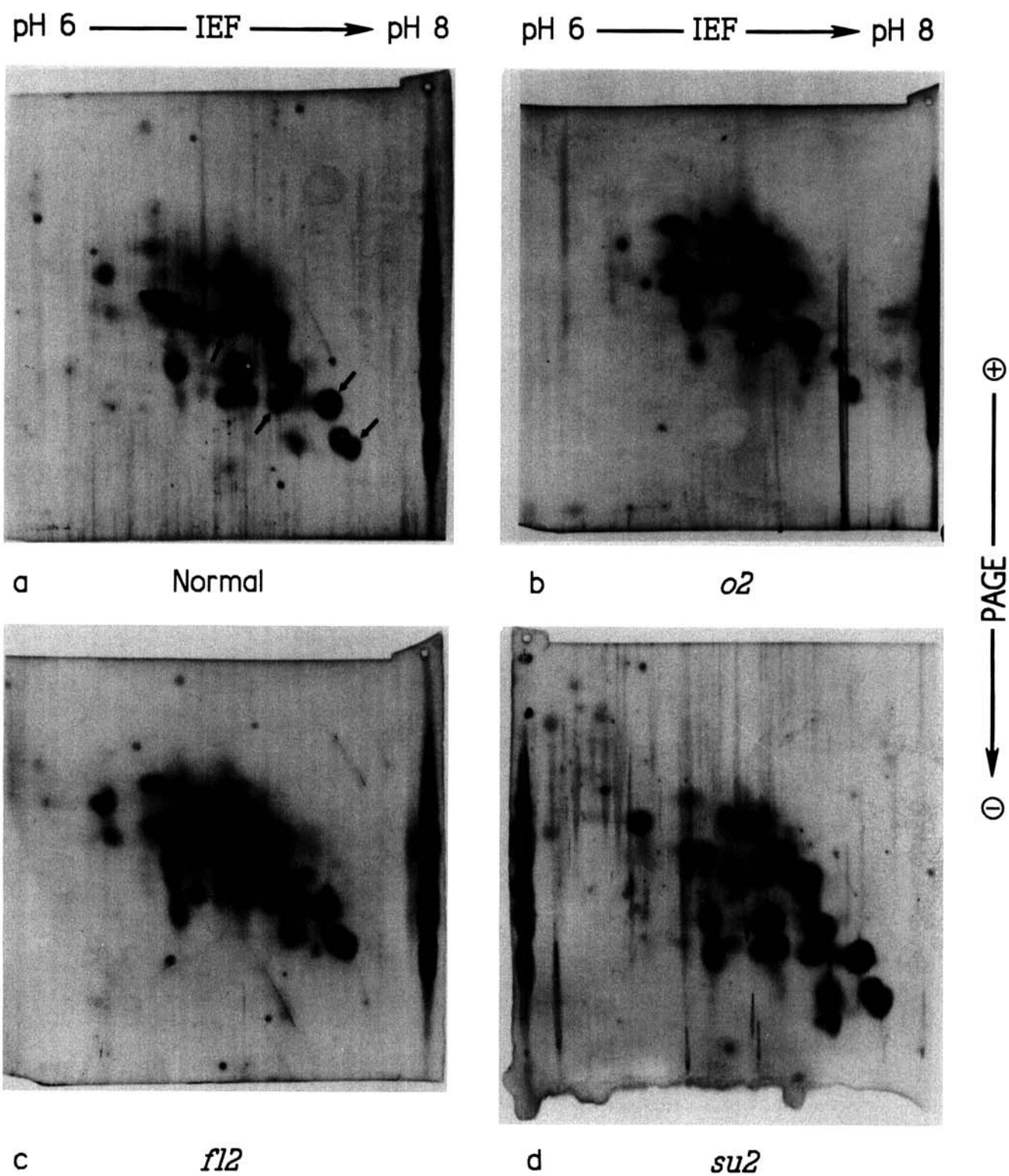

Fig. 7. Two-dimensional electrophoresis patterns (first dimension, isoelectric focusing, pH 6-8; 2nd dimension, pH 3.5 aluminum lactate polyacrylamide gel electrophoresis) of zeins from **a,** W64A normal; **b,** *opaque-2*; **c,** *floury-2*, and **d,** *sugary-2* endosperms of mature grains. Arrows in **a** indicate major components absent in *opaque-2* W64A zein, **b**.

contained more ASGs than zein from normal endosperms; this may represent the reduced level of zein in these extracts, so that traces of ASGs become more prominent. Zein heterogeneity is not fully apparent in these SDS-PAGE patterns, because of the high concentration of protein applied and the overlap of proteins between classes due to slight differences in molecular weight and amino acid sequence.

SDS-PAGE patterns of alcohol-insoluble glutelins in mature maize grain were reported earlier by Paulis et al (1975). Patterns of these proteins (extracted with pH 8.2 borate buffer containing 0.5% SDS and 2% 2-ME) throughout development are shown in Figure 6. Normal and *o2* endosperm extracts are similar at each stage of grain development; in *o2* extracts at 30 and 48 days, bands at mol wt 35,000 (arrows) may be more prominent than in normal extracts. Most bands intensify during maturation of each genotype, but some became less prominent, such as those in 18- and 22-day extracts of mol wt 33,000 (indicated by arrows).

Two-Dimensional Electrophoresis of Zeins

The heterogeneity of zein is not fully apparent upon either electrophoresis or IEF in a single direction because of the multiplicity of zein components and their similarity. Two-dimensional electrophoresis (IEF followed by SDS-PAGE) was used by Hagen and Rubenstein (1980) to more clearly show differences between zeins from normal and *o2* endosperms. However, optimum resolution is not attained in this system, because many zeins have similar molecular weight. IEF followed by aluminum lactate PAGE better resolves zeins in normal corns (Wall et al 1984); patterns vary with inbred lines. Figure 7 compares zeins of mature normal and *o2* W64A separated by this system. A number of zeins in W64A normal (indicated by arrows) were absent in W64A *o2*. These zeins may be the higher molecular weight species, but current information (Viotti et al 1982) suggests that some lower molecular weight zeins may also not be expressed in *o2*. Zeins are also reduced in the *fl2* mutant (Nelson et al 1965), but there is no significant difference in the two-dimensional electrophoresis pattern of W64A *fl2* and normal zeins (Fig. 7). Apparently the *fl2* gene reduced all zeins to the same degree (Fig. 7), as also indicated by zein SDS-PAGE patterns (Lee et al 1976). Similarly, the *su2* gene, which has a lesser effect in reducing endosperm zein content, also reduced all zeins to the same extent (Fig. 7).

CONCLUSION

Despite the much higher concentrations of albumins and globulins in W64A *o2* than in normal endosperm, there were few differences in component polypeptides of each of these protein classes between these two genotypes, as evidenced by SDS-PAGE. A prominent 37,000 mol wt albumin band in normal W64A was absent in *o2*; this protein may be identical to the B32 protein isolated by Soave et al (1981) that has been implicated as a possible regulator of protein synthesis. This band remains very intense at all stages of normal grain development. The disappearance of other albumins and globulins during development may be caused by insolubilization by disulfide bond formation in the maturing kernel. Such proteins may contribute to glutelin formation.

The floury structure of the *o2* endosperm differs significantly from that of the normal vitreous endosperm. The *o2* endosperm has more matrix protein as observed by scanning electron microscopy of destarched endosperms (Wolf et al 1967). The amount of alcohol-insoluble glutelin is also much higher in the *o2* than in the normal kernel. If alcohol-insoluble glutelin is the major component of matrix protein, it does not increase resistance of the *o2* kernel to breakage, a major problem for high lysine grains. The decrease in zein and changes in protein distribution may be major factors contributing to kernel softness.

Two-dimensional electrophoresis of normal and *o2* zeins indicates a large number of genetic variants in both genotypes. However, certain zeins are absent in the *o2* mutant line, indicating that the regulatory element initiating their production is absent or is malfunctioning. Further understanding of how the normal allele *O2* functions to cause maximum zein production and vitreous endosperm formation would be valuable to help breeders produce grain with optimum nutritional quality and acceptable grain properties.

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