Embryo-Specific Globulins from *Zea mays* L. and Their Subunit Composition

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The globulin fraction is a major constituent of the protein in mature embryos of maize. In inbred line A188 this protein fraction can be separated by gel filtration chromatography into two fractions. One is a very large aggregate of subunits of mostly 63 000 daltons with some subunits of 65 000 and 71 000 daltons. The second fraction has a molecular weight of about 127 000 and includes major subunits of 50 000, 29 000–30 000, and 21 000–22 000. During development the 71 000-, 63 000-, and 50 000-dalton polypeptides appear earlier than the others. Certain inbred lines also contain a polypeptide of 58 000 daltons. The overall proportions of these polypeptides differed somewhat among several inbred and hybrid cultivars examined. However, these polypeptides were not observed in globulin preparations from vegetative tissues. When an antiserum prepared against the total globulin fraction was tested against extracts from vegetative tissue, no positive reaction could be detected by using immunodiffusion tests. Therefore, it appears that occurrence of these proteins in diploid sporophytic tissues is limited to the developing embryo.

The storage proteins of maize are an area of great interest from the standpoints of seed quality and nutrition. Of these proteins, the zeins of the endosperm have received the greatest attention from biochemists and molecular biologists. This attention is primarily due to the high proportion of zeins in the grain and their deficiency of certain essential amino acids, lysine and tryptophan in particular. However, the globulins of the embryo are also of interest because they constitute about 5–8% of the grain protein and are relatively rich in lysine and arginine (Wall and Paulis, 1978). Moreover, wet milling processes have made corn germ available as a feed supplement.

We know as yet little of the metabolism of embryo globulins during seed develoment and germination, but it is likely that they serve an important function as nitrogen reserves since globulins are present in both monocot and dicot embryos. The scutellum contains most of this globulin, probably within the protein bodies of this organ (Wall and Paulis, 1978). The accumulation of albumins plus globulins during seed development takes place within 5–6 weeks after pollination and then it halts (Moureaux and Landry, 1972; Khavkin et al., 1977). Mobilization of this reserve occurs early during germination (Khavkin et al., 1978).

Although maize albumins are electrophoretically quite heterogeneous, only a few native globulin species are extracted with salt [see Wall and Paulis (1978) for a review]. Danielson (1949) reported two globulin components with sedimentation rate constants of 2.6 and 8.5 S and corresponding molecular weights of 26 000 and 166 000. The native globulins were heterogeneous by starch gel electrophoresis, including perhaps nine major components at acid pH (Wall and Paulis, 1978). These authors reported a different pattern at alkaline pH.

Paulis et al. (1975) identified four prominent globulin subunits in extracts from endosperms using $NaDodSO_4$ gel electrophoresis (62, 41, 25, and 12 kilodaltons). Khavkin et al. (1978) reported nine prominent subunits (68, 60, 56, 52, 49, 25.5, 24.5, 23, and 21 kilodaltons) in globulin extracted from the embryo. The Khavkin group also identified an acidic (pH 4.7 insoluble, legumin-like) fraction enriched in the 68 000- and 60 000-dalton subunits and an acid-soluble (vicilin-like) fraction enriched in the 60 000-dalton polypeptide and the remaining lower molecular weight subunits. They prepared an antiserum against the total globulin fraction and determined immunologically that globulins begin to accumulate between 18 and 27 days after pollination (Khavkin et al., 1978). Dierks-Ventling and Ventling (1982) have reported that the endosperm globulins have the same subunit composition as found in the embryo. They reported about 12 major polypeptides with sizes ranging from about 10 000 to 80 000 but did not tabulate their molecular weights.

The objective of the study presented here was to describe the organization and subunit structure of the embryo globulins of inbred line A188, which is one of the principal lines employed in our tissue culture studies. This provides a basis for using these developmentally regulated proteins as tissue-specific markers to investigate the developmental pathway of plants regenerating from cultures of this genotype. Here we demonstrate the presence of two native forms of globulin in normal embryos of A188. We also show that (with the exception of endosperm tissues cited above) both of these forms are embryo specific, in agreement with the results of Khavkin et al. (1978).

EXPERIMENTAL SECTION

Preparation of Embryo Globulin. Our methods were adapted from the classic extraction procedures of Osborne and collaborators [see Wall and Paulis (1978) for a summary]. Dry seeds were soaked in water overnight at 5 °C and were dissected into embryo and endosperm portions with a razor. In some cases embryos were extracted from dry seeds. No proteolysis could be detected as being caused by this soak. The embryos (or other tissues) were then freeze-dried and pulverized with the aid of liquid nitrogen. The power was extracted 3 times with petroleum ether (or acetone at 5 °C for seedling tissues) to remove lipids and pigments. In some cases the solvent contained phenylmethanesulfonyl fluoride (10 mM) as a protease inhibitor (Gardner et al., 1971) with no discernible effect on the results. Larger quantities of freeze-dried embryos were homogenized directly with petroleum ether by using a Polytron.

The residual solvent was evaporated from the powder with N_2, and the powder was extracted with 0.50 M NaCl plus 14 mM 2-mercaptoethanol in a glass–glass hand homogenizer. The insoluble material was centrifuged out at 5000*g* and the pellet extracted and centrifuged again. The combined supernatants were then centrifuged for 30 min at 30000*g* and the lipid pellicle was wiped off of the top if possible. The soluble protein was then dialyzed extensively against distilled water at 5 °C to precipitate the globulins. Completion of dialysis was estimated with

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a Beckman conductivity meter. The contents of the dialysis tubes were centrifuged at 30000*g* for 15 min, and the supernatant (albumin fraction) was pipetted off. The pellet (globulin fraction) was resuspended in 0.5 M NaCl (usually containing 3 mM NaN_3 as a preservative) and stored at 5 °C.

Gel Electrophoresis. These procedures were derived from those of O'Farrell (1975) and Laemmli (1970). Before electrophoresis, the albumin and globulin proteins were precipitated in 3 volumes of 95% ethanol and centrifuged in a Beckman microfuge. The protein was then dissolved in the solubilizing reagent appropriate for the type of electrophoresis. For sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis in one dimension in slabs, the sample buffer consisted of 0.15 M Tris-HCl (pH 8.8), 1 mM Na_2EDTA, 1% $NaDodSO_4$ (Sigma, lot no. 106C-0188), 20% glycerol, and 0.001% bromphenol blue (all w/v) and 1% 2-mercaptoethanol (v/v) added just before use. The sample to be used for isoelectric focusing (IEF) was first dissolved in freshly prepared 6 M urea plus 1% 2-mercaptoethanol (v/v).

The isoelectric focusing gel included a 39:1 ratio of acrylamide to bis(acrylamide) and a total monomer concentration of 4.0%. Gels also contained 5% glycerol, 0.2% NP-40 (Sigma), and 0.0008% riboflavin 5-phosphate (all w/v) and a mixture of 3-10, 4-6, and 5-7 Biolytes (Bio-Rad Laboratories), 1.33:1:1 (v/v), total Biolyte concentration 5% (v/v) or 2% based on solids content. This mixture of ampholytes was found by trial to give a good separation of the proteins in corn embryos. The mixture was adjusted to pH 5.5 with 1 N HCl, and 125-mm gels were photopolymerized in 3.5 mm i.d. × 180 mm tubes. Samples were layered over the gels and focused at 5 °C from the alkaline end for 15–18 h at 500 V after an initial prefocusing of about 30 min at about 100–200 V.

Slab gels (110 × 151 × 1.2 mm) for $NaDodSO_4$–polyacrylamide gel electrophoresis were formed at 15% (for some one-dimensional work) or with 10–20% linear gradients of acrylamide. The ratio of acrylamide to bis(acrylamide) was 75:1. Gels also included 0.375 M Tris-HCl (pH 8.8), 0.10% $NaDodSO_4$ (w/v), 0.03% ammonium persulfate (w/v), and 0.025% Temed (*N,N,N′,N′*-tetramethylethylenediamine). The stacking gel was formed with 4.5% total acrylamide, 0.125 M Tris-HCl (pH 5.7), 0.1% $NaDodSO_4$ (w/v), 0.10% ammonium persulfate (w/v), and 0.10% Temed. For two-dimensional work, the isoelectric gel was extruded from the tube with gentle pressure and layered over the stacking gel. The gel was sealed to the slab with 1% (w/v) low electroendosmosis agarose (Sigma type I) in stacking gel buffer. Gels were run at 12 mA/gel at room temperature until the tracking dye penetrated the running gel 1 cm and 24 mA/gel thereafter.

Slab gels were gently removed from the plates, placed in a glass baking dish, and fixed in three changes of 10% 2-propanol–10% acetic acid (v/v) on a rotary shaker to remove $NaDodSO_4$. Two-dimensional gels were then stained on a shaker overnight in Coomassie Brilliant Blue R 0.04%, Crocein Scarlet 0.05%, and $CuSO_4$ 0.5% (all w/v) dissolved in 27% 2-propanol plus 10% acetic acid (v/v). Gels were destained in fixative on a shaker. One-dimensional slab gels were fixed and stained as above, but the stain did not include Crocein Scarlet and $CuSO_4$.

Immunological Methods. Antiserum was prepared against total A188 globulin by injections of 4–5 mg of protein mixed with Freund's complete adjuvant (first injection) or incomplete adjuvant (later injections) into several sites in the back muscle of a rabbit at 2-week intervals. Adjuvant was a product of Miles. Four injections were required to obtain antiserum with good titer. The course of antibody production was monitored by the method of double immunodiffusion in Nobel agar (Difco) plus 0.5 M NaCl, 10 mM $CaCl_2$, and 3 mM NaN_3. Wells (4-mm diameter and about 40-μL volume) were spaced 10 mm apart in a 3–4-mm layer of agar. Incubations were run at room temperature and observed at daily intervals. When a high titer serum was obtained, the γ-globulin (IgG) fraction was prepared by precipitation with 50% saturated $(NH_4)_2SO_4$. The precipitated protein was dissolved in 3 mM NaN_3 (about 20 mg/mL protein) and stored at 5 °C. It was generally used without dilution.

Transfer of Proteins to Nitrocellulose Paper. One milligram of globulin from A188 was labeled with dansyl chloride, and the unreacted dye was removed as described by Talbot and Yphantis (1971). The protein was then precipitated with 2 volumes of ethanol and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis as described above, except that a one-slot sample comb was used so that the same bands ran completely across the gel. After the gel was photographed under 366-nm illumination, it was sliced longitudinally into three pieces. One part was fixed and stained with Brilliant Blue R as usual. The polypeptides of the other pieces were transfered to nitrocellulose paper using a blotting technique similar to that described by Renart et al. (1979). The blotting was done at 5 °C with 0.5 M NaCl. Under these conditions less than half of the protein transferred to the paper as judged from fluorescence. Control experiments demonstrated that untreated globulins and globulins carbamoylated with KOCN (1 M at 50 °C, 1 h) transfered from $NaDodSO_4$–polyacrylamide gel electrophoretic gels to nitrocellulose paper with equal efficiency and gave identical sharp patterns when stained on the paper. The stain was Amido Black [0.5% w/v, in 27% 2-propanol and 10% acetic acid (both v/v), destained with the same solvent] since Brilliant Blue R binds irreversibly to the nitrocellulose. Therefore uncarbamoylated globulins were used for the transfer experiments. A comparison of potential blotting solutions showed that 0.5 M NaCl and 0.15 M Tris-HCl, pH 8.8, gave similar results.

Localization of Antigens on Nitrocellulose Paper. This procedure is a modification of that described by Towbin et al. (1979). After blot transfer the nitrocellulose papers were then incubated with antibody solution for 1 h at room temperature. The incubation solution contained rabbit globulin IgG (0.2 mg/mL), plus bovine serum albumin (10 mg/mL) to prevent nonspecific binding of IgG to the nitrocellulose in 0.15 M NaCl–3 mM sodium azide. After this incubation the papers were briefly rinsed and then washed twice for 30 min in saline alone. The IgG which bound to the paper was then labeled by incubation for 1 h with peroxidase-conjugated goat anti-rabbit IgG (Sigma, 0.033 unit/mL in saline). The excess second antibody was removed by two washes in saline, and the antibody-binding bands were stained by incubation for 5–10 min in substrate [0.01% diaminobenzidine (w/v) and 0.01% H_2O_2 (v/v) in 0.1 M potassium phosphate buffer, pH 7.0]. This substrate gave more satisfactory results than the *o*-dianisidine used by Towbin et al. (1979). All incubations were at room temperature on a slow rotary shaker.

RESULTS AND DISCUSSION

Subunit Composition and Molecular Weight. Two-dimensional gel electrophoresis demonstrated that a great many distinct polypeptides occurred in the 0.5 M M NaCl soluble fraction of mature corn embryo proteins (Figure 1a,b). However, most of these polypeptides were

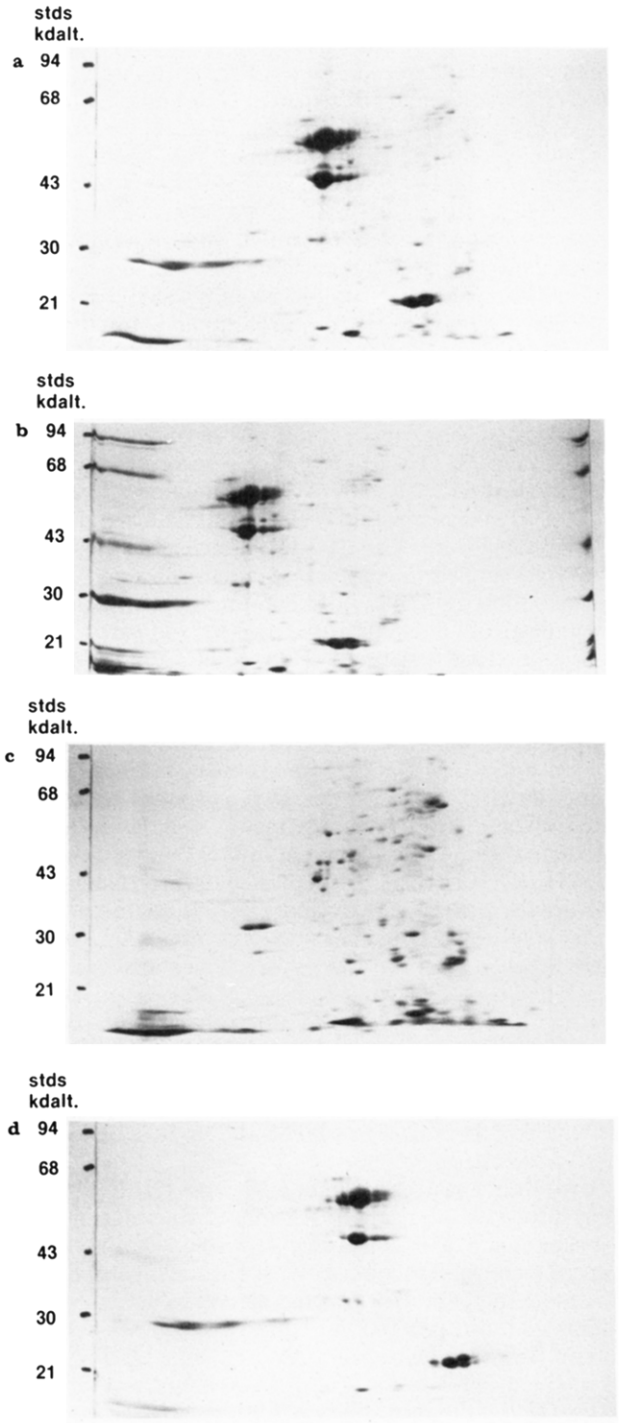

Figure 1. Two-dimensional gel electrophoresis patterns of embryo proteins of the corn inbred A188. The first dimension (isoelectric focusing) is left (alkaline origin) to right (acidic). $NaDodSO_4$ gel electrophoresis was from top to bottom. (a) Total salt extract (500 μg of protein loaded). (b) Same as (a) with molecular weight standards added to the edges of the $NaDodSO_4$ gel for standardization: 90, 68, 43, 30, 21, and 14 kilodaltons. (c) Albumins (500 μg of protein). (d) Globulins (150 μg of protein).

contributed by the H_2O-soluble (albumin) fraction (Figure 1c). The globulin fraction had a much simpler pattern (Figure 1d). This fraction contained about eight groups of polypeptides, each of which appeared to contain several minor charge modifications of polypeptides of the same molecular weight. The major size classes in mature A188 embryos were 65, 63, 50, 30, 29, 22, and 21 kilodaltons. Of these the 65K and 63K groups clustered closely together, as did the 20K and 29K groups and the 22K and 21K

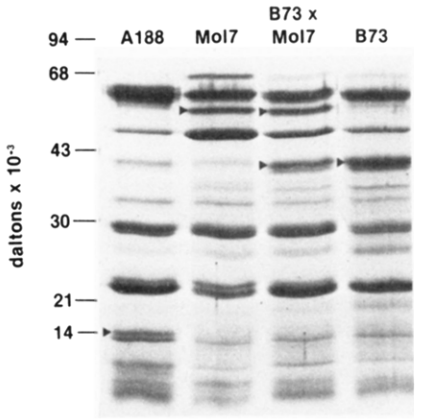

Figure 2. A comparison of the globulin patterns of inbred lines and their hybrids. The load was 75 μg/lane. Lanes (left to right): standards; A188; Mo17; B73 × Mo17; B73.

groups. Whether these groupings represent some precursor–product or gene–family relationships is not known.

Several other common inbred lines and hydrids were also examined by one- and two-dimensional gel electrophoresis. A one-dimensional gel is presented in Figure 2 for comparative purposes. Several of the inbred lines and hybrids contained a polypeptide family of 58 000 daltons in addition to the polypeptides present in A188. Some inbreds differed in their content of certain polypeptides: a polypeptide family of 42 000 daltons was prominent in B73 and its hybrids, while a 14 000-dalton polypeptide was readily apparent only in A188. The other differences among lines were in the relative proportions of the constituent polypeptide families. Hybrids tended to exhibit a simple additive inheritance of these polypeptide families. It is not known if this pattern of inheritance is correlated with any phenotype of the inbreds or the resulting hybrids.

Danielsson (1949) and Khavkin et al. (1978) reported that the globulin fraction they isolated contained two proteins. According to Khavkin et al. (1978), one of these proteins was rich in 68 000- and 60 000-dalton polypeptides. This could correspond to our polypeptide families of 71 000 and 63 000 or 65 000 daltons, respectively. The other protein they studied was rich in polypeptides of 60 000 daltons and lower. We therefore attempted to fractionate our globulin preparation by molecular weight on agarose gel (Bio-Gel Al.5 m) or HPLC using Waters I-60, I-125, I-250 columns in series. Both methods yielded an elution profile with two peaks. The lower molecular weight peak was estimated to be 127 000 daltons from the calibration of the Waters columns (Figure 3), while the molecular weight of larger peak exceeded the calibration curves of both columns and could only be estimated as being above 200 000 daltons.

Alternate fractions from the A1.5 m column were subjected to $NaDodSO_4$ gel electrophoresis. The results (Figure 4, fractions 13–15 vs. 23–27) showed that there is no overlap in the polypeptide families present in the large aggregate (Figure 4, fractions 13–15), which contained primarily the three largest polypeptide families (63 000, 65 000, and 71 000 daltons) with traces of many others, while the 127 000-dalton protein contained the smaller families (Figure 4, fractions 23–27). Some of the larger polypeptides appeared to tail off the large peak, as if they were released during chromatography. The migration rate of the "tailing" peptides was constant, suggesting that this putative disaggregation did not involve proteolysis.

The subunit composition (50 000, 29 000–30 000, and 21 000–22 000 daltons, respectively) of the 127 000-dalton protein suggests a ratio of one molecule of 50 000 daltons

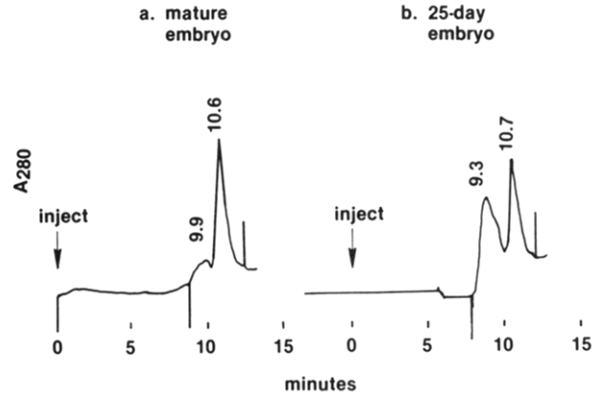

Figure 3. Chromatography of A188 embryo globulin on HPLC–gel filtration columns. Fifty microliters (180 μg) of proteins was chromatographed in 0.1 M potassium phosphate, pH 7.2, at a rate of 2 mL·min^{-1}. Calibration of this column series with bovine serum albumin, its dimer, ovalbumin, and guanosine gave a log–linear line with $r^2 = 0.998$. The second peak is about 127 000 daltons; the first peak is >200 000 daltons using this regression line.

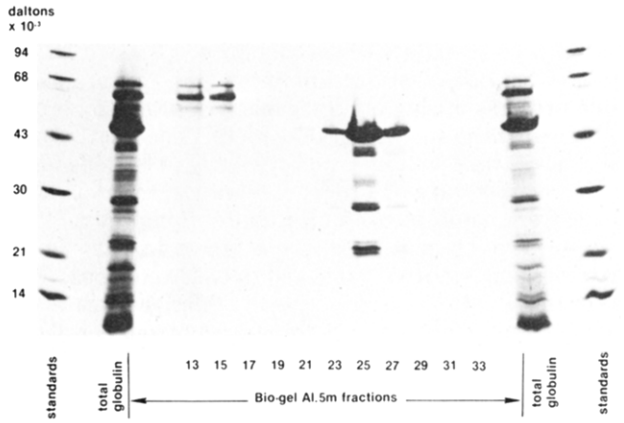

Figure 4. NaDodSO$_4$–polyacrylamide gel electrophoresis patterns of globulin mature embryos of A188 that were chromatographed on a column of Bio-Gel A1.5 m and 1-mL fractions were collected. Alternate fractions, beginning with fraction 11 (second fraction before the void volume), were then analyzed by NaDodSO$_4$ gel electrophoresis as described under Experimental Section. Lanes: (1) standards; (2) blank; (3) unfractionated globulin; (4–15) column fractions; (16) unfractionated globulin; (17) blank; (18) standards.

plus one or two of each smaller subunit per complex. It is not known whether the polypeptide families in the large aggregate (Figure 4, fractions 13–15) associate with each other or if they form large, separate aggregates. Since the 0.5 M concentration of the NaCl used in the extraction and column solutions was arbitrary, it is possible that higher NaCl concentrations or other dissociating agents would have resulted in the dissociation of this aggregate to a protomer.

Developmental Specificity of Globulin. Sodium dodecyl sulfate–polyacrylamide gel electrophoretic profiles of globulins extracted from 10-day old seedling tissues did not exhibit detectable levels of the embryo globulins, except in the extract of residual seed scutellum (Figure 5). The residual scutellum did show a band coincident with the 63 000-dalton band; however, its identity was not established. Otherwise, the pattern suggested extensive degradation of the globulin in this organ and the presence of many new proteins.

Since identity cannot be established by gel electrophoresis, a specific immune reagent was needed to provide more information on the tissue distribution of these proteins. Therefore, antibody was prepared to the total globulin fraction of mature A188 embryos. This serum was found to react with all the principal polypeptides of this fraction. This was determined by transferring the electrophoretically separated polypeptides (prelabeled with dansyl chloride) from an NaDodSO$_4$ slab gel (one dimensional) to nitrocellulose paper by a blotting technique (Renart et al., 1979). Since the proteins were prelabeled with dansyl chloride, it was possible to confirm that all major bands were transferred to the paper. The nitrocellulose paper was then treated with the antiserum γ-globulin fraction, washed extensively with saline, and then treated with peroxidase-conjugated goat anti-rabbit IgG. The immune-reactive bands were then stained with diaminobenzidene plus H_2O_2. All transferred polypeptides were found to react in fair proportion (as judged by eye) to their concentration as revealed by protein staining in the original gel and by the intensity of the transferred fluorescent bands (not shown).

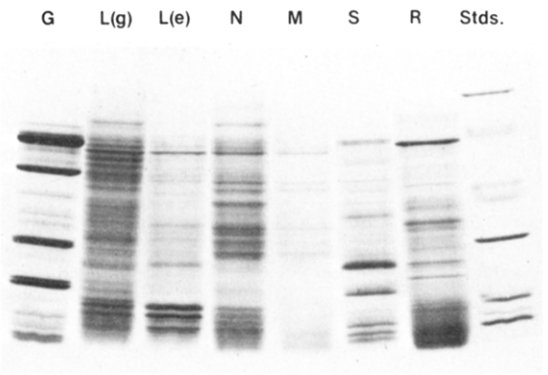

Figure 5. NaDodSO$_4$–polyacrylamide gel electrophoresis of globulins extracted from tissues of A188. Lanes (left to right): (G) from mature embryo, remaining lanes from 7-day seedlings: L(g), (green) leaf; L(e), (eliolated) leaf; N, coleoptile node; M, mesocotyl; S, (residual) scutellum; R, root; Stds, standards.

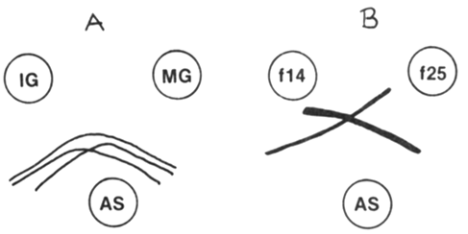

Figure 6. Double-immunodiffusion tests of anti-A188 globulin serum. The γ-globulin (IgG) fraction from the immune serum was in the central well (AS). (A) Reactions with globulin from MG (mature embryos of A188) and IG [immature (25 days postpollination] embryos of A188. (B) Reactions with fractions from the column of Bio-Gel A1.5 m (Figure 4): f14, high molecular weight aggregate; f25, 127 000-dalton peaks.

Using a double-immunodiffusion technique it was also determined that the serum reacted with both protein peaks from the A1.5 m gel (Figure 4) and not with other fractions. Two or three precipitin arcs could be seen in the unfractionated globulin from mature embryos of Al88 (Figure 6A). Immature embryos of A188 (25 days postpollination) showed the same precipitin lines, except that one appeared to be present at higher concentration in the immature embryo globulin than in the extract of mature embryos (Figure 6A), since the arc was displaced toward the antiserum well in the extract of immature material.

Double-immunodiffusion tests were performed to confirm the specificity of the immune serum. The same globulin preparations from vegetative tissues (analyzed by

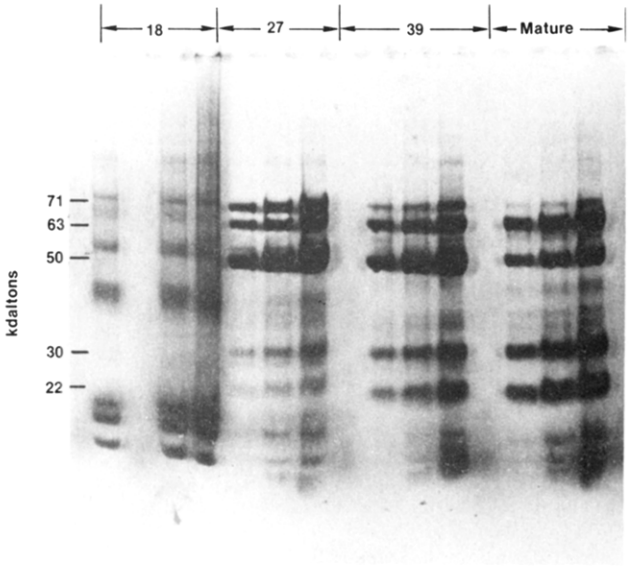

Figure 7. Changes in polypeptide composition of A188 embryo globulin during development. Embryos were excised from fertilized ears of A188 from different intervals. Each globulin fraction was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis at three different concentrations (50, 100, and 200 μg of protein). The bands are less sharp than in previous experiments because a stacking gel was omitted. Left to right: 18-, 27-, and 39-day embryos and mature embryo from dry seed.

electrophoresis in Figure 5) were used for the tests. The results are given in Table I. The only seedling tissue to react was the weak reaction obtained with the extract from the residual scutellum of the 10-day-old seedling. These qualitative results are in line with those of Khavkin et al. (1978), who showed rapid loss of globulin from the scutellum during germination.

Other data indicate that the globulins in the high molecular weight aggregate (Figure 4, fraction 14) do not cross-react with those of the 127 000-dalton fraction from the Bio-Gel column (Figure 6B, fraction 25). This result indicates that the lower molecular weight fraction is not a degradation or processing product of the large aggregate.

Since we had established the embryo specificity of the globulins, it was of interest to determine the time of origin of these polypeptides. The rate of increase of globulin in A188 was found to be greatest between 19 and 25 days postpollination and to be essentially complete sometime between 25 and 40 days (data not shown); this is in agreement with results from previous investigations (Wall and Paulis, 1978).

Sodium dodecyl sulfate gel electrophoresis of the globulin proteins of the developing embryo shows that the globulins extracted at 18 days are not typical of the mature pattern (Figure 7). However, the pattern at 27 days is typical of that found later, except for the proportions of the subunits. The 71 000-dalton polypeptides are a much higher proportion in other genotypes (Figure 2). Moreover, the polypeptides of 21–22 and 29–30 kilodaltons appear

Table I. Reactions of Antiserum to A188 Embryo Globulin[a]

experiment	antigen source	protein fraction	reaction
I	A188 mature embryo	globulin	+
		albumin	–
	immature embryo (15 days)	globulin	+
		albumin	–
II	B73 × Mo17 mature embryo	globulin	+
III	A188 seedling (7 day)	salt	
	leaf (green or etiolated)	extracts	–
	mesocotyl		–
	scutellum		+ (weak)
	root		–

[a] All reactions are double-immunodiffusion tests. A positive reaction is scored as one or more precipitin arcs. Each well contained about 200–500 μg of protein to ensure a positive reaction, if possible. A188 embryo globulin gave a positive reaction with 1.8 μg of protein. Thus, a protein making up as little as 1% of the total could be detected.

to increase relatively later than the others, particularly between 27 and 39 days. After 39 days their proportion is close to that in the mature embryo.

These results demonstrate that maize embryos contain two embryo-specific storage globulins. Each of these proteins contains distinct subunits. The appearance of these proteins during embryogenesis appears to be programmed by the stage of development. The relative proportions of these subunits are very likely to be dependent on the relative stage of development of the embryo. If so, this pattern could be useful for determining the relative stage of development of embryos grown in vitro or for characterizing embryos produced in tissue cultures. The use of these patterns to describe the relative stage of development of embryos will be the subject of another paper.

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