Structure and Origin of Maize Endosperm Alcohol-Insoluble Glutelin

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The composition, structure, and origin of maize endosperm alcohol-insoluble glutelin polypeptides were explored. A pH 8.2 borate buffer containing sodium dodecyl sulfate (SDS) extracted only small amounts of this protein, but addition of 2% 2-mercaptoethanol (ME) increased the yield considerably. SDS-polyacrylamide gel electrophoresis indicated that glutelins extracted in the absence of ME are high molecular weight aggregates of subunits linked by disulfide bonds. The amount of glutelin extractable with borate buffer containing SDS and ME increases and its composition changes during kernel maturation. The possibility that this glutelin originated by disulfide cross-linking of soluble proteins was evaluated by comparing their two-dimensional electrophoretic patterns to those of albumins and globulins and examining the immunochemical interaction of glutelins with rabbit antibodies against endosperm globulin proteins.

Since the studies of Osborne (1897), maize (*Zea mays* L.) endosperm proteins have been classified as follows: albumins, soluble in water; globulins, soluble in saline solution; zein, soluble in 70% ethanol; glutelins, soluble in dilute alkali (when extracted in that sequence). Foster et al. (1950) demonstrated that most maize proteins, including glutelin, could be dissolved in detergent solution containing a reducing agent such as sulfite. Boundy et al. (1967) found that glutelin extracted by alkali could be reduced with 2-mercaptoethanol (ME) in 8 M urea to cleave disulfide bonds and release polypeptides that could be separated by starch gel electrophoresis. After extracting albumins, globulins, and zeins from endosperm, Nielsen et al. (1970) concentrated glutelin in the residual endosperm by digesting starch with α-amylase. Paulis and Wall (1971) dissolved most glutelin by reducing disulfides and alkylating resulting glutelin sulfhydryls in the presence of 6 M guanidine hydrochloride. They separated reduced-alkylated proteins into a 70% ethanol-soluble fraction and one requiring 6 M guanidine hydrochloride for solution. Landry and Moureaux (1970) fractionated glutelin proteins in a simpler manner by successive extraction with 55% 2-propanol plus 0.6% ME; pH 10 borate buffer + 0.6% ME; and pH 10 borate buffer + 0.5% sodium dodecyl sulfate (SDS) + 0.6% ME.

Fractionated glutelin components differ greatly in electrophoretic properties and amino acid compositions. Alcohol-soluble reduced-alkylated glutelins have amino acid compositions resembling those of zeins, with low amounts of lysine and tryptophan but high contents of leucine, glutamine, and proline. They have relatively low molecular weight (MW) as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Paulis et al., 1975). Alcohol-soluble glutelins can be further fractionated into water-soluble and -insoluble fractions; SDS-PAGE showed the water-soluble fraction to have MW 27000, and the water-insoluble fraction contained MW 18000, 15000, and 10000 components. Complementary DNA, coding for the MW 15000 water-insoluble protein, was cloned and sequenced by Pedersen et al. (1986) and that for the MW 27000 water-soluble protein by Prat et al. (1985). These proteins are located in protein bodies, along with zein

(Pedersen et al., 1986; Ludevid et al., 1984).

In contrast to knowledge of alcohol-soluble glutelins, information available about alcohol-insoluble glutelins remains sparse. Unlike alcohol-soluble glutelins, these proteins have amino acid compositions rich in lysine and tryptophan (Paulis and Wall, 1971), similar to salt-soluble proteins; these proteins correspond to the fraction V or G3 in the Landry and Moureaux (1970) fractionation scheme. Starch gel electrophoresis in pH 3.2 aluminum lactate buffer (Paulis and Wall, 1971) and SDS-PAGE (Paulis et al., 1975) reveal alcohol-insoluble glutelin to be heterogeneous in charge and molecular weight. Christianson et al. (1969) isolated particulates termed glutelin I from homogenates of immature endosperms by sedimentation in a sucrose gradient; these particulates had an amino acid composition similar to alcohol-insoluble glutelin but different from that of protein bodies. In mature maize kernels, M. J. Wolf (Northern Regional Research Center, Peoria, IL, 1981, personal communication) found that residual matrix protein in amylase-destarched 70% ethanol-extracted endosperm sections was disrupted by buffered SDS + ME solutions. Matrix proteins bind starch and protein bodies within the endosperm.

Thus, alcohol-insoluble glutelin is important to the physical properties and utilization of maize grain. It contributes lysine and tryptophan, which occur in low concentration in maize. Indeed, increased alcohol-insoluble glutelin (with decreased zein) accounts for the improved nutritional quality of *opaque*-2 grain (Paulis et al., 1969). By forming a rigorously associated matrix, alcohol-insoluble glutelin immobilizes starch and protein in hydrated meal, necessitating alkaline treatment to form a dough. Alcohol-insoluble glutelin also prevents easy separation of starch and protein during maize wet-milling; lengthy steeping in SO_2 solution is required to facilitate that process.

Recently, Godwin and Satterlee (1985) reported that most maize endosperm glutelin can be solubilized by extended stirring in buffered SDS solutions without reducing agent. This observation indicates that the glutelin matrix might not be extensively cross-linked by disulfide bonds and might be associated mainly by hydrophobic interactions. This concept could lead to novel methods for processing maize. The present work was undertaken to investigate the nature of linkages between alcohol-insoluble glutelin polypeptide chains and to gain better insight into the proteins involved. The origin of glutelin was also explored by examining changes in amount and composition of components in developing maize endosperm and by

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comparing endosperm proteins using electrophoretic and immunochemical techniques.

MATERIALS AND METHODS

Corn Extract Preparations. Golden Harvest 2480 maize was grown from hybrid seed (Sommer Seed Co., Pekin, IL) near Princeville, IL. W64A inbred seed was purchased from Illinois Foundation Seeds Inc., Champaign. Grains were dry-milled at room temperature by a conventional laboratory process; endosperm used for protein separations from these grains was from the first- and third-break grit (Brekke et al., 1973), which was rapidly ground in a Udy Cyclone Mill through a 0.24-mesh screen. To study changes in protein composition during grain development, W64A was grown in a field plot at NRRC and self-pollinated; ears were harvested 18, 22, 30, and 48 days after pollination. The germ and pericarp were hand-dissected from these grains; the endosperm was lyophilized, weighed, and ground in a Udy mill.

Ground samples (20 g) were defatted by stirring with 200 mL of cold 82% hexane + 18% methanol (v/v) for 1 h at 4 °C. After being filtered on a Buchner funnel, solids were reextracted with 100 mL of solvent for 45 min at 4 °C. The filtered meal was air-dried.

To extract globulins, albumins, and nonprotein nitrogen (NPN), 5 g of ground endosperm was extracted twice with 50 mL of cold 0.5 N NaCl at 4 °C for 1 h with magnetic stirring. After 20-min centrifugation at 25400g in a Beckman L8-70 M ultracentrifuge, supernatants were combined. Residues were extracted twice with 100 mL of 70% ethanol + 0.5% sodium acetate at room temperature by stirring 2 h.

Centrifuged residues were next extracted by one of two sequential extraction schemes. In the first sequence, residues were extracted for designated times at room temperature with 50 mL of 0.025 M borate buffer + 0.5% SDS, adjusted to either pH 8.2 or 10 with NaOH; each extraction was performed twice. Residues after centrifugation were extracted twice for 2 h with 50 mL of the same buffer containing 2% ME. In the second extraction sequence, residues were first extracted twice at room temperature by stirring for 2 h with 70% ethanol + 0.5% sodium acetate + 2% ME and then twice for 2 h at room temperature with 50 mL of 0.025 M borate buffer containing 0.5% SDS and 2.0% ME at either pH 8.2 or 10.

Yields of protein in extracts were determined by Kjeldahl nitrogen analysis. NPN in the 0.5 N NaCl extract was determined by taking an aliquot, adding an equal volume 20% trichloroacetic acid (TCA), shaking, and centrifuging the protein precipitate. The supernatant was extracted with an equal volume of diethyl ether to remove TCA. Both protein precipitate and aqueous NPN solution were analyzed for nitrogen. Remaining extracts (ca. 80 or 180 mL) were dialyzed against 4 L of distilled water at 4 °C for 48 h with four changes of water. The dialyzed 0.5 N NaCl extracts were centrifuged to partially separate soluble albumins from globulins. All extracts and solids were lyophilized and weighed.

Electrophoresis and Isoelectric Focusing. SDS-PAGE was conducted in a vertical Hoefer SE600 apparatus with 1.5-mm gels. The running gel was made with 10% or 14% monomer (2.7% of total monomer was bisacrylamide) in 0.3 M Tris-Cl buffer, pH 8.8; the stacking gel contained 0.125 M Tris buffer (pH 6.8) and 4% total monomer, 2.7% of which was bis. Gels were polymerized with tetramethyethylenediamine and potassium persulfate. Protein samples (2 mg) were dissolved in 0.5 mL of treatment buffer (0.063 M Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% ME, 0.01% bromphenol blue) and heated at 100 °C for 90 s, and 20 μL was applied to each well. Standard reference proteins from Bio-Rad (Richmond, CA) of low molecular weight [lysozyme (14400); soybean trypsin inhibitor (21500); carbonic anhydrase (31000); ovalbumin (45000); bovine serum albumin (66200); phosphorylase B (92500)] and high molecular weight [ovalbumin (45000); bovine serum albumin (66200); phosphorylase B (92500); β-galactosidase (116250); myosin (200000)] were introduced into adjacent wells. Tank buffer was 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. Electrophoresis was conducted at 30-mA constant current per gel until marker dye was 1 cm from gel bottom (approximately 3 h). Gels were stained overnight with 0.06% Coomassie Blue R250 in 30% methanol, 10% TCA, and 60% water and destained with 50% methanol, 40% water, and 10% acetic acid.

Isoelectric focusing (IEF) (Wall et al., 1984) was carried out on an LKB Multiphor (Model 2117) horizontal electrophoresis unit with 27 × 11.5 × 0.2 cm 5% polyacrylamide gels that contained 2% LKB ampholines (pH 3.5–10) and 8 M urea. Samples reduced with ME and alkylated with acrylonitrile (6 mg of protein/100 mL) according to Paulis and Wall (1977) were absorbed on 1.0 × 0.5 cm cambrele wicks, and IEF was conducted at 4 °C at 13-W constant power for 4 h. Gels were silver-stained with the kit of Bio-Rad according to the manufacturer's directions for 2-mm gels.

Two-dimensional electrophoresis combined IEF and SDS-PAGE as follows: For application to the IEF gel, the protein concentration for reduction and alkylation was increased to 20 mg/0.1 mL of 8 M urea. After IEF, a strip (4.0 mm wide and 9.5 cm long) was cut from the center of the desired gel lane and equilibrated with 20 mL of SDS-PAGE treatment buffer for 20 min at room temperature. The SDS-PAGE gel was 2 mm thick, with 10% acrylamide in the running gel. The 5% acrylamide stacking gel contained two slots, one 11 cm long and 1 cm deep to accommodate the IEF strip and the other 0.5 cm wide to hold protein or standard solutions equilibrated with treatment buffer. The gel strip was sealed in place with 1% agarose dissolved in pH 6.8 Tris stacking gel buffer with 0.1% SDS. Gels were overlaid with tank buffer, run at 40 mA per gel until the dye was 1 cm from bottom (approximately 3 h), and stained with silver stain.

Western Immuno-blot Transfer Detection of Proteins on Gels. Rabbit anti-maize globulin antibodies were prepared by injection of 1.3 mg of crude globulin from W64 maize dissolved in 2 mL of 50% Freunds adjuvant emulsion, followed by three similar booster injections at 2-week intervals. Two weeks later, 20 mL of blood was withdrawn, allowed to clot, and centrifuged to obtain antiserum. Proteins were transferred from SDS-PAGE gels to nitrocellulose sheets (Towbin et al., 1979) with a Bio-Rad 49-BR Transblot Cell filled with 80% 25 mM Tris + 192 mM glycine buffer, pH 8.3, plus 20% methanol. The gel was placed adjacent to the nitrocellulose sheet, sandwiched between filter papers and fiber pads in the holder, and oriented so the gel was toward the cathode. Duplicate transfers were carried out overnight at 30 V and 0.1 A at room temperature. One nitrocellulose transfer was stained for protein with 0.1% amido black in 45% methanol–10% acetic acid, and the other was tested for reaction with rabbit antibodies to maize globulins. Bio-Rad goat anti-rabbit horseradish peroxidase conjugate (GAR-HRP) was used according to the manufacturer's instruction. Nonspecific binding to nitrocellulose membranes was blocked with gelatin. The membranes were then washed, incubated with a 1:200 dilution of rabbit antiglobulin serum in buffer,
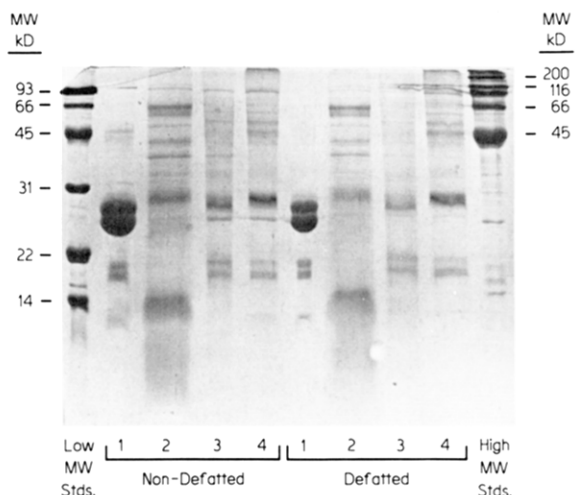
Figure 1. Effect of defatting on SDS-PAGE separations of proteins in extracts from maize endosperm: 1, zein; 2, 0.5 N NaCl extract; 3, 0.5% SDS in pH 10 borate buffer; 4, 0.5% SDS + 2% ME in pH 10 borate buffer.

washed, exposed to GAR-HRP solution, washed, and reacted with hydrogen peroxide and 4-chloronaphthol to visualize sites of antibody–protein association.

RESULTS AND DISCUSSION

Extraction of Glutelin Proteins. Table I summarizes yields of saline-soluble, zein, and glutelin protein obtained by sequential extraction of endosperm meals from Golden Harvest 2480 corn. Glutelins were extracted with buffers containing SDS, with or without ME, under various conditions of pH, time, temperature, and defatting. Defatting slightly increased yield of salt-soluble proteins (column 1) but had little effect on zein yields. Following removal of saline and ethanol solubles, pH 10 borate buffer containing 0.5% SDS extracted only 10.8% of the remaining protein (column 2). Slightly less protein was obtained from defatted meal (column 1). Some protein dissolved by SDS may be associated with lipid in nondefatted endosperm. When stirring time with pH 10 buffer containing 0.5% SDS was increased to 2 h (column 3), yield of protein doubled. Decreasing the SDS-containing buffer to pH 8.2 diminished the yield of protein extracted in two consecutive 1-h stirrings to 6.1% (column 4); higher pH might disrupt disulfide bonds. Extraction at pH 8.2 for 4 h two times increased the yield of protein, compared to extraction for 2 h, to 9.2% (column 5), possibly due to rupture of disulfide bonds by shearing during stirring. Mechanical and chemical factors may also be responsible for increased protein yield when the pH 8.2 SDS buffer extraction is performed at 50 °C (column 6). The yields of protein from two successive ME + SDS buffer extractions are such that the sum of the sequential extractions with and without ME in the SDS buffers is fairly constant (40.2–46.5%). This indicates that harsher conditions (high pH and prolonged stirring) diminish yields of protein extracted readily by SDS + ME. The ease and rapidity with which glutelins are solubilized by SDS + ME at pH 8.2 suggests that most glutelin proteins are cross-linked by intermolecular disulfide bonds. It is noteworthy that the total yield of protein extracted in each instance by SDS and SDS + ME in buffered media equals that yielded by the sequence 70% ethanol + 0.5% sodium acetate followed by SDS + ME in alkaline buffer (column 7).

Figure 1 compares SDS-PAGE patterns of proteins extracted from nondefatted and defatted corn endosperm. Proteins in zein and 0.5 N NaCl fractions are similar, but a MW 27 000 band in the buffered SDS and buffered SDS
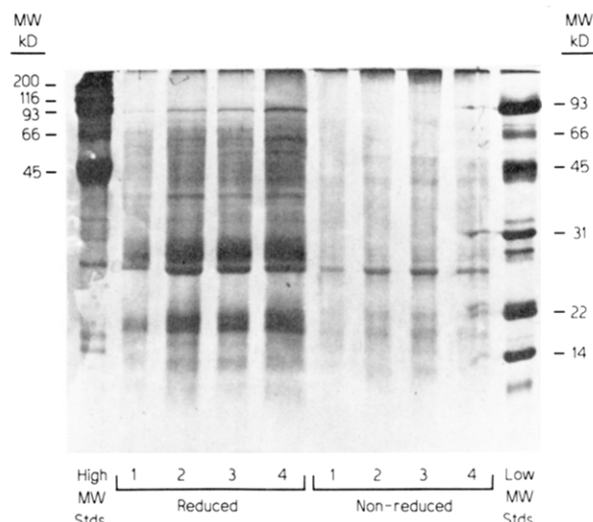
Figure 2. SDS-PAGE of reduced and nonreduced glutelin proteins extracted with 0.5% SDS-containing buffers under various conditions: 1, pH 10 borate buffer, 1 h, room temperature, 2×; 2, pH 8 borate buffer, 1 h, room temperature, 2×; 3, pH 8 borate buffer, 4 h, room temperature, 2×; 4, pH 8 borate buffer, 1 h, 50 °C, 2×.
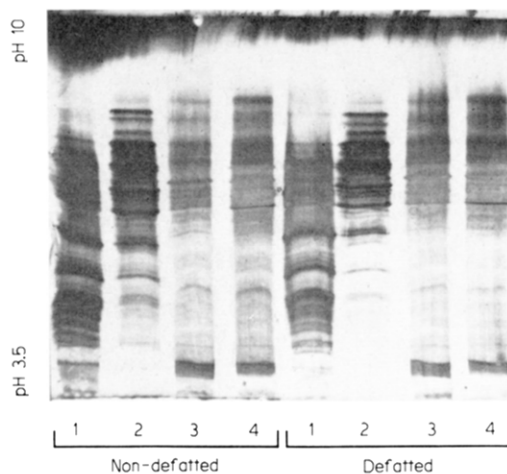
Figure 3. Isoelectric focusing patterns of protein extracts from nondefatted and defatted endosperms of maize: 1, 0.5 N NaCl solubles; 2, zein; 3, 0.5% SDS + pH 10 borate buffer; 4, 0.5% SDS + pH 10 borate buffer + 2% ME.

+ ME extracts is less apparent in the same defatted endosperm extracts. Different extraction conditions had little effect on composition of SDS and SDS + ME extracts of glutelins, as indicated by SDS-PAGE (data not shown). Despite higher yields of protein at pH 10, and with extraction for longer periods and at higher temperature, the SDS-PAGE patterns of all SDS extracts were similar, as were those obtained with SDS and ME. Polypeptides with mobilities similar to those of zein appear in the buffered SDS extract, while polypeptides with mobilities similar to those of alcohol-soluble glutelins are present in the buffered SDS + ME extracts (Figure 1). Even though stirring with SDS disperses significant amounts of protein, SDS-PAGE in the absence of reducing agent indicates that proteins in these extracts are high-MW entities. As shown in Figure 2, almost none of the proteins migrate into SDS-PAGE gels without prior reduction (no ME in buffer). An exception is the band of MW ≈27 000 (also noted in Figure 1); this is possibly a membrane protein absent in defatted endosperm that may require SDS for dissociation from lipid. However, after reduction with ME, most

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Table I. Percent of Total Protein Extracted from Corn Endosperm[a] under Various Conditions of Defatting, pH, Time, and Temperature

extraction procedure	extraction sequence						
	1	2	3	4	5	6	7
0.5 N NaCl, 10 v/w, 1 h, 4 °C, 2×	4.80	4.10	4.30	4.19	4.22	4.22	4.32
70% ethanol + 0.5% sodium acetate, 20 v/w, RT, 2 h, 2×	43.6	46.8	45.3	43.7	42.5	46.8	45.2
70% ethanol + 0.5% sodium acetate + 2% ME, 10 v/w, RT, 2 h, 2×							17.2
pH 10 borate buffer + 0.5% SDS, 10 v/w, RT, 1 h, 2×	10.1	10.8					
same as above but 2 h, 2×			19.1				
pH 8.2 borate buffer + 0.5% SDS, 10 v/w, RT, 1 h, 2×				6.1			
same as above but 4 h, 2×					9.2		
pH 8.2 borate buffer + 0.5% SDS, 10 v/w, 50 °C, 2×						13.7	
pH 8.2 or 10 borate buffer + 0.5% SDS + 2% ME, RT, 2 h, 2×	36.1	31.2	26.8	40.4	37.3	26.5	22.4
total extracted	94.6	92.2	95.2	94.4	93.8	91.2	89.1

[a] Extraction sequence 1 was performed on defatted meal (7.97% total protein); all other extraction sequences used nondefatted meal (7.47% total protein).

SDS-extracted glutelin migrates into the SDS-PAGE gel to give discrete bands.

Figure 3 shows IEF patterns of reduced and alkylated 0.5 N NaCl, 70% ethanol + 0.5% sodium acetate, 0.5% SDS buffer soluble, and 0.5% SDS + 2% ME buffer soluble proteins from nondefatted and defatted endosperms. IEF patterns of SDS and SDS + ME extracts are qualitatively similar, providing further evidence that polypeptides solubilized by these two extractants are the same but that they differ in degree of disulfide cross-linking.

Formation of Glutelin during Grain Development. Since glutelin in mature maize endosperm appears to be disulfide cross-linked, the question arises as to whether disulfide bond formation occurs just after glutelin polypeptide synthesis or later during maturation. To study this question, W64A grain was harvested at intervals after pollination and protein fractions were extracted from the endosperms. Our results for saline-soluble proteins and zein (Table II) are consistent with the findings of Murphy and Dalby (1971); however, different extraction reagents and conditions were used to study glutelin. The percentages of total NPN and salt-soluble proteins decline markedly during kernel maturation (Table II). Since total protein per endosperm increases considerably during kernel development, the absolute decrease in NPN per endosperm is not as marked (Table II). A highly significant decrease in amount of salt-soluble proteins per endosperm occurs. Zein first increases greatly with stage of development, both as percent of protein and in amount per endosperm, but levels off after 30 days. In extraction sequence 1, both SDS + buffer and SDS + ME + buffer fractions of glutelin increase during development in amount per milligram of endosperm tissue (Table II), but SDS + buffer fractions appear to level off after 30 days. The SDS + ME + buffer fraction, however, shows a larger increase during the last period (30–48 days), indicating that upon maturation and dehydration a more extensive glutelin matrix forms. In extraction sequence 2, the solvent 70% ethanol + 0.5% sodium acetate extracts increased amounts of protein up to 30 days; after 30 days, the rate of increase slows. Amounts of protein in subsequent SDS + buffer + ME extracts continue to increase to 48 days.

To determine whether these changes in amounts of different proteins during kernel development are due to specific polypeptides, as previously investigated by Di Fonzo et al. (1977), isolated albumin, globulin, zein, ethanol-soluble reduced glutelin (extracted with ethanol + 0.5% sodium acetate + 2% ME), and alcohol-insoluble reduced glutelin (extracted with 0.5% SDS + pH 8.2 borate + 2% ME) fractions from endosperms of different stages were subjected to SDS-PAGE (Figures 4 and 5).
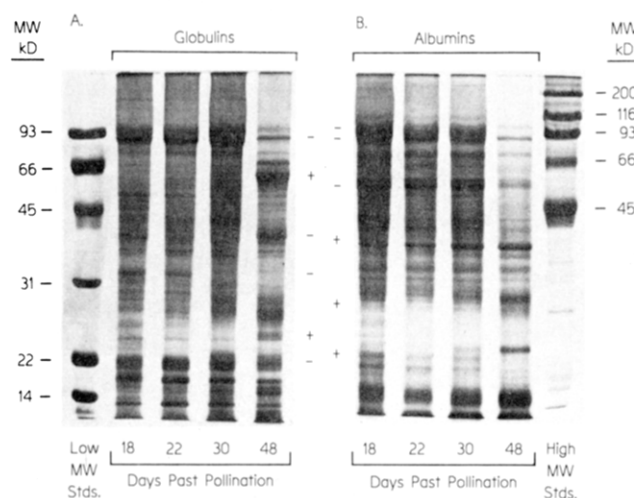
Figure 4. Changes in compositions of (A) globulins and (B) albumins in developing W64A maize endosperm as determined by SDS-PAGE.
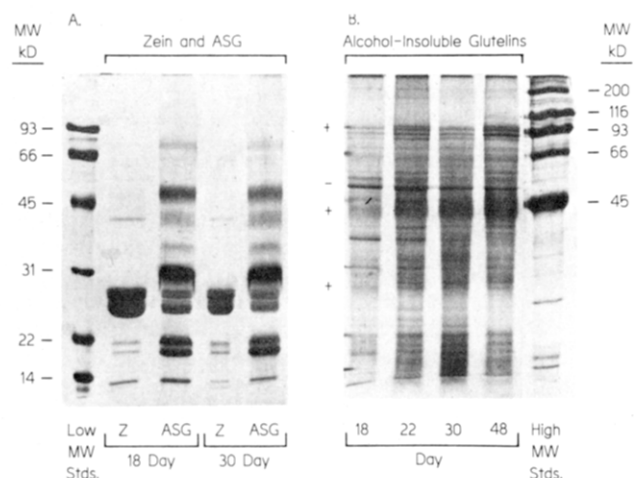

Figure 5. Changes in compositions of zeins, alcohol-soluble glutelins, and alcohol-insoluble glutelins in endosperms of developing kernels of W64A maize as determined by SDS-PAGE: Z, zein; ASG, alcohol-soluble glutelin.

Significant changes occur during maturation. Certain globulins (indicated by minus signs) become reduced in intensity during maturation and are faint at 48 days (Figure 4A). Similarly, some albumins become less pronounced as maturity progresses (minus signs, Figure 4B), but others become more prominent (plus signs). Zeins and alcohol-soluble reduced glutelins retain fairly constant compositions (Figure 5A). Certain alcohol-insoluble glutelin polypeptides increase in relative amount during

Table II. Changes in Proteins of W64A Corn Endosperm at Different Stages of Development

	harvest time, days after pollination			
	18	22	30	48
% protein in endosperm	17.1	16.5	14.8	15.3
endosperm wt, mg	36.7	51.5	93.5	100.6
protein per endosperm, mg	6.2	8.5	13.8	15.4

	% total protein			
	18 days	22 days	30 days	48 days
nonprotein nitrogen	16.6 (1.0)[a]	9.9 (0.8)	7.3 (1.0)	3.9 (0.6)
protein extractant				
0.5 N NaCl	27.7 (1.8)	18.0 (1.5)	12.8 (1.6)	4.0 (0.6)
70% ethanol + 0.5% NaOAc	35.7 (2.9)	41.8 (3.6)	58.4 (8.5)	61.5 (9.4)
sequence 1				
0.5% SDS + pH 8.2 borate buffer	5.1 (0.3)	7.0 (0.8)	9.6 (1.3)	10.0 (1.7)
0.5% SDS + pH 8.2 borate buffer + 2% ME	11.1 (0.7)	15.0 (1.3)	12.4 (1.8)	18.5 (3.3)
sequence 2				
70% ethanol + 0.5% NaOAc + 2% ME	7.7 (0.5)	10.2 (0.9)	6.5 (1.1)	8.3 (1.3)
0.5% SDS + pH 8.2 borate buffer + 2% ME	12.2 (0.8)	14.8 (1.3)	14.0 (2.0)	19.0 (2.9)

[a] Values in parentheses indicate milligrams per endosperm.
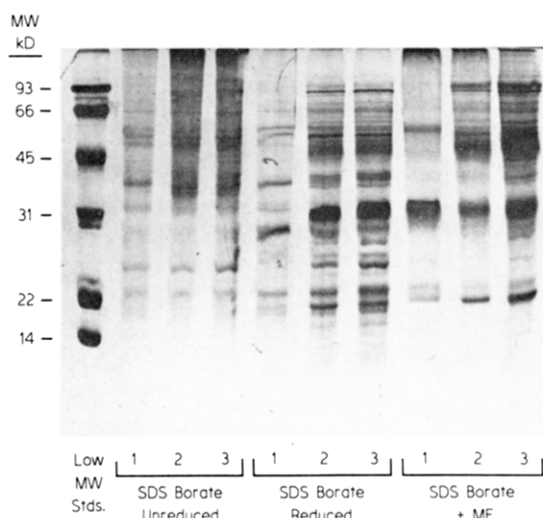
Figure 6. SDS-PAGE patterns (unreduced and reduced) of glutelins extracted with SDS + borate buffer from endosperms of developing W64A maize kernels, compared to proteins extracted with SDS + borate buffer + ME: 1, 18 days; 2, 30 days; 3, 48 days after pollination.

maturation. Some of these polypeptides have apparent molecular weights similar to those albumins and globulins that decreased in 40-day endosperm extracts, as indicated by minus signs. Possibly certain globulins and albumins become denatured during final stages of maturation and form disulfide linkages, thus becoming incorporated into glutelin; however, further evidence is required to support such a conjecture.

Figure 6 shows SDS-PAGE patterns of proteins extracted from developing kernels with pH 8.2 borate buffer containing 0.5% SDS. Even at 18 days little free polypeptide occurs in unreduced extract. More protein migrates into the gel from unreduced extracts of 30- and 48-day endosperm extracts, but bands are diffuse and much protein remains at the origin. When the 18-day extract is reduced, the same bands are present as in the nonreduced 18-day extract, indicating that the small amount of glutelin extracted with SDS + borate buffer at the early stage is not extensively disulfide-linked. In contrast, the reduced SDS + borate extracts of 30- and 48-day endosperms show considerably more protein bands of higher intensity, indicating that at this stage even lyophilized grain extracts have soluble glutelin with extensive disulfide cross-linking. The SDS + borate + ME extracts

contain bands similar to those in the SDS + borate extracts at 30 and 48 days, indicating similarity of these glutelin proteins. Many bands present in glutelins of these older samples are absent at 18 days, indicating that they are incorporated into glutelin at a later developmental stage.

Similarities between Glutelin Polypeptides and NaCl-Soluble Proteins. Two-dimensional electrophoresis provides a more discerning approach to the elucidation of relationships among polypeptides in different extracts of maize. Globulins, albumins, and alcohol-insoluble glutelins from mature endosperm were resolved by IEF in the first direction and SDS-PAGE in the second (Figure 7). All extracts have distinctly different compositions. Globulins contain groups of proteins (arrows 1–3) that appear unique to that fraction. Some lower MW proteins (arrows 4) may be similar in globulins and albumins. Since separation of albumins by precipitation of globulins upon dialysis against water is incomplete, some cross contamination was expected. One protein type (labeled 5) is present only in albumins, but not in the other extracts. Globulins, albumins, and glutelins all contain a series of proteins (labeled 6) with similar molecular weights but varying pI. To establish whether these proteins are identical, they must be isolated and further characterized. Alcohol-insoluble glutelin also contains high-MW proteins (labeled 7) not present in albumin or globulin. Since these proteins were derived from mature endosperm, they may not reflect changes in composition of albumins and globulins during grain development. High-MW components are present at early developmental stages (30 days postpollination) and may contribute to the group 7 proteins of mature endosperm.

Another approach to investigating relationships among different protein groups is examination of their immunological properties. To determine whether antibodies to globulins interact with glutelin polypeptides, serum containing rabbit antibodies to maize endosperm globulins was reacted with protein fractions separated by SDS-PAGE. A membrane, containing transferred separated alcohol-insoluble glutelin, alcohol-soluble glutelin, zein, albumins, and globulins from W64A endosperm (Figure 8A), was stained with amido black (left); on the right, protein–antibody complexes were detected by peroxidase-conjugated goat anti-rabbit antibody serum and the color reaction was catalyzed by the peroxidase. Positive signals indicated that the globulin antibodies complexed with specific proteins in all fractions except the zein extract. The MW 27 000 alcohol-soluble glutelin protein reacted with globulin an-
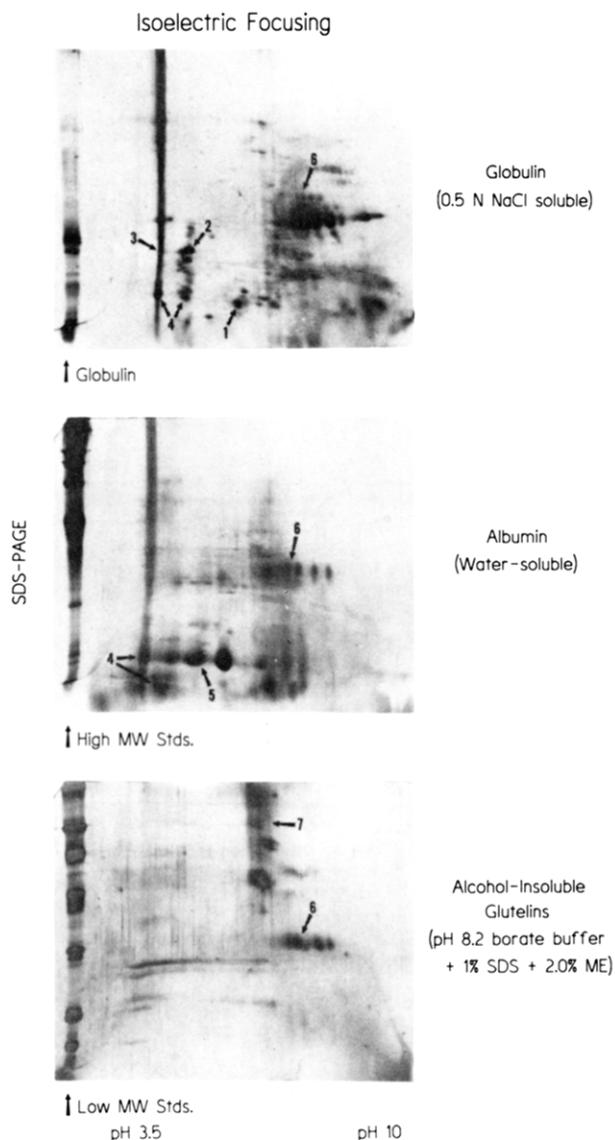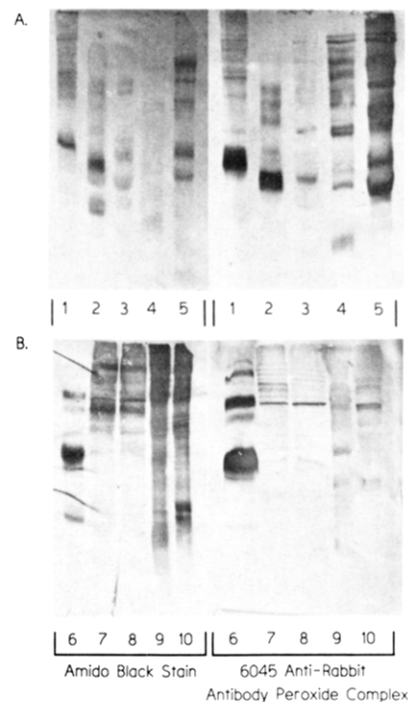
Figure 7. Two-dimensional electrophoresis of globulins, albumins, and alcohol-insoluble glutelins from mature maize endosperms.

Figure 8. Detection of maize globulin–antibody complexes with extracted maize proteins transferred from SDS gels onto nitrocellulose: left, amido black stain of nitrocellulose transferred proteins; right, goat anti-rabbit antibody peroxidase detected globulin antibody–protein complexes. (A) 1, SDS + ME + pH 8.2 buffer extract; 2, ethanol-soluble reduced glutelin; 3, zein; 4, albumins; 5, globulins. (B) 6, 22-day endosperm alcohol-soluble glutelin; 7, 48-day endosperm alcohol-insoluble glutelin; 8, 22-day endosperm alcohol-insoluble glutelin; 9, 22-day endosperm albumins; 10, 22-day endosperm globulins.

tibodies, indicating common immunological determinants. Albumins with molecular weights similar to those of specific globulins also associated strongly with globulin antibodies.

The reaction of globulin antibodies with proteins from W64A at various stages of development was also investigated (Figure 8B). The 22-day alcohol-soluble glutelin reacted strongly with globulin antibodies, indicating further that these proteins have common determinants at all development stages. The 48-day alcohol-insoluble glutelin had more bands that react with globulin antibodies than did the 22-day alcohol-insoluble glutelin, but both alcohol-soluble and -insoluble glutelin at 48 days had a band that reacted strongly with the globulin antibody. This band was not obvious in the 22-day albumin–antibody association pattern, which had another prominent antibody–protein reaction band.

CONCLUSIONS

These studies support earlier work that indicated that glutelin proteins are linked by disulfide bonds and that a portion of these proteins are in a matrix engulfing protein bodies and starch granules. The findings of Godwin and Satterlee (1985) that these proteins can be dissolved after extensive stirring in the presence of SDS in pH 10 buffer may be explained by physical disruption of the disulfide-linked matrix and by the known action of elevated pH in catalyzing disulfide breakdown (Sanderson et al., 1978). Extending the stirring time and elevating the pH increased the amount of protein dispersed by SDS. The fact that the protein dispersed in SDS after prolonged stirring consists of high molecular weight disulfide-linked polypeptide aggregates, as shown by SDS-PAGE in nonreducing and reducing media, supports the idea that disulfide bonds are important parts of glutelin structure. Separate experiments have shown that pH 8 buffer with ME in the absence of SDS failed to dissolve much glutelin. This finding suggests that glutelin proteins are unfolded and associate by hydrophobic interactions as well as through disulfide bonds. Landry and Moureaux (1970) dissolved appreciable protein with pH 10 buffer and ME in the absence of SDS. A small portion of glutelin protein is soluble in SDS without ME at pH 8. These proteins may be associated with lipids in membranes since they are not present in glutelin from maize endosperm previously extracted with solvents to remove polar lipids.

In maize, precursors to alcohol-insoluble glutelin proteins may be deposited in unstable vacuolar type protein bodies (distinct from those containing zein) or in the cytoplasm where they interact in the developing and dehydrating kernel to yield a disulfide-linked matrix. The idea that alcohol-insoluble glutelin proteins are derived from globulin or albumin-like proteins is an attractive hypothesis. As shown here and by others (Moureaux and Landry, 1972; Murphy and Dalby, 1971), the amount of globulin and albumin proteins per endosperm diminishes as maturation progresses. Also, the amino acid composition of alcohol-insoluble glutelin is similar to that of salt-soluble proteins (Wall and Paulis, 1978). Sodium fluoroacetate inhibits

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albumin and globulin synthesis in corn seeds, but glutelin deposition continues, indicating that glutelin is synthesized from existing protein (Zel'ko and Kalinnikov, 1970). Kalinnikov and Tolokonnikov (1971) observed that glutelin production in corn grain is inhibited by iodoacetate, which alkylates SH groups; they suggested that glutelin is formed by disulfide linkage of soluble proteins. However after reduction of disulfide bonds, the alcohol-insoluble glutelins are best solubilized in solutions containing SDS, indicating that they must have more exposed hydrophobic regions than native globulins or albumins.

Our efforts to identify albumin or globulin proteins as participants in glutelin formation by Western blotting were not conclusive. The immunological experiments showed a broad range of interaction of maize globulin antibodies against globulins, albumins, alcohol-soluble glutelin, and alcohol-insoluble glutelin proteins. It is probable that these protein fractions contain individual polypeptides that are evolutionarily related and are derived from a common precursor, as suggested for endosperm proteins of barley (Kreis et al., 1985). Further isolation of individual albumin, globulin, and glutelin polypeptides, as well as sequence analyses, is needed to fully define structural relationships.

LITERATURE CITED

Boundy, J. A.; Woychik, J. H.; Dimler, R. J.; Wall, J. S. "Protein Composition of Dent, Waxy, and High-amylose Corns". *Cereal Chem.* **1967**, *44*, 160–169.

Brekke, O. L.; Griffin, E. L.; Shove, G. C. "Dry Milling of Corn Artificially Dried at Various Temperatures". *Trans. ASAE* **1973**, *16*, 761–765.

Christianson, D. D.; Nielsen, H. C.; Khoo, U.; Wolf, M. J.; Wall, J. S. "Isolation and Chemical Composition of Protein Bodies and Matrix Proteins in Corn Endosperm". *Cereal Chem.* **1969**, *46*, 372–381.

Di Fonzo, N.; Fornasari, E.; Salamini, F.; Soave, C. "SDS-Protein Subunits in Normal, *Opaque-2* and *Floury-2* Maize Endosperms". *Maydica* **1977**, *22*, 77–88.

Foster, J. F.; Yang, J. T.; Yui, N. H. "Extraction and Electrophoretic Analysis of the Proteins of Corn". *Cereal Chem.* **1950**, *27*, 477–487.

Godwin, G. J.; Satterlee, L. D. "Solubility of Maize Endosperm Glutelins Without Reduction in Buffers Containing Detergent". *Cereal Foods World* **1985**, *30*, 550.

Kalinnikov, D. D.; Tolokonnikov, V. I. "Effect of Respiration Inhibitors on Protein Synthesis in Ripening Maize Grain". *Fiziol. Rast.* **1971**, *18*, 1147–1151.

Kreis, M.; Forde, B. G.; Rahman, S.; Miflin, B. J.; Shewry, P. R. "Molecular Evolution of the Seed Storage Proteins of Barley, Rye and Wheat". *J. Mol. Biol.* **1985**, *183*, 499–502.

Landry, J.; Moureaux, T. "Hétérogénéité des Glutélines du Grain de Maïs: Extraction Sélective et Composition en Acides Aminés des Trois Fractions Isolées". *Bull. Soc. Chem. Biol.* **1970**, *52*, 1021–1037.

Ludevid, M. D.; Torrent, M.; Martinez-Izquierdo, J. A.; Puig-

domenech, P.; Palau, J. "Subcellular Localization of Glutelin-2 in Maize (*Zea mays* L.) Endosperm". *Plant Mol. Biol.* **1984**, *3*, 227–234.

Moureaux, T.; Landry, J. "La Maturation du Grain de Maïs. Evolution Qualitative et Quantitative des Différentes Formes Azotées". *Ann. Physiol. Veg.* **1972**, *10*, 1–18.

Murphy, J. J.; Dalby, A. "Changes in the Protein Fractions of Developing Normal and *Opaque-2* Maize Endosperm". *Cereal Chem.* **1971**, *48*, 336–349.

Nielsen, H. C.; Paulis, J. W.; James, C.; Wall, J. S. "Extraction and Structure Studies on Corn Glutelin Proteins". *Cereal Chem.* **1970**, *47*, 501–512.

Osborne, T. B. "The Amount and Properties of the Proteids of the Maize Kernel". *J. Am. Chem. Soc.* **1897**, *19*, 525–532.

Paulis, J. W.; Wall, J. S. "Fractionation and Properties of Alkylated-Reduced Corn Glutelin Proteins". *Biochim. Biophys. Acta* **1971**, *251*, 57–69.

Paulis, J. W.; Wall, J. S. "Comparison of the Protein Compositions of Selected Corns and Their Wild Relatives, Teosinte and *Tripsacum*". *J. Agric. Food Chem.* **1977**, *25*, 265–270.

Paulis, J. W.; James, C.; Wall, J. S. "Comparison of Glutelin Proteins in Normal and High-Lysine Corn Endosperms". *J. Agric. Food Chem.* **1969**, *17*, 1301–1305.

Paulis, J. W.; Bietz, J. A.; Wall, J. S. "Corn Protein Subunits: Molecular Weights Determined by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis". *J. Agric. Food Chem.* **1975**, *23*, 197–201.

Pedersen, K.; Argos, P.; Naravana, S. V. L.; Larkins, B. A. "Sequence Analysis and Characterization of a Maize Gene Encoding a High-Sulfur Zein Protein of M_r 15,000". *J. Biol. Chem.* **1986**, *261*, 6279–6284.

Prat, S.; Cortadas, J.; Puigdomenech, P.; Palau, J. "Nucleic Acid (cDNA) and Amino Acid Sequences of the Maize Endosperm Protein Glutelin-2". *Nucleic Acids Res.* **1985**, *13*, 1493–1504.

Sanderson, J.; Wall, J. S.; Donaldson, G. L.; Cavins, J. F. "Effect of Alkaline Processing of Corn on its Amino Acids". *Cereal Chem.* **1978**, *55*, 204–213.

Towbin, H.; Staehelin, T.; Gordon, J. "Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications". *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350–4354.

Wall, J. S.; Paulis, J. W. "Corn and Sorghum Proteins". In *Advances in Cereal Science and Technology 2*; Pomeranz, Y., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1978; pp 135–219.

Wall, J. S.; Fey, D. A.; Paulis, J. W.; Landry, J. "Improved Two-Dimensional Electrophoretic Separation of Zein Proteins: Application to Study of Zein Inheritance in Corn Genotypes". *Cereal Chem.* **1984**, *61*, 141–146.

Zel'ko, A. A.; Kalinnikov, D. D. "Biosynthesis of Protein Fractions in Corn". *Mater. Nauchn. Konf.—Voronezh. Skh. Inst. im. K. D. Glinki* **1970**, *1*, 70–72.