Isolation and Chemical Composition of Protein Bodies and Matrix Proteins in Corn Endosperm

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ABSTRACT

Microscopic examination of endosperm cells reveals the presence of starch granules and protein bodies held together by a protein matrix. To separate these subcellular structures, endosperm tissue, obtained 24 days after pollination, was gently homogenized in phosphate buffer. The particulate cell components were then separated by zone sedimentation on a sucrose density gradient. Sequential fractions were taken from different depths of the gradient tube, analyzed for protein, and examined microscopically. Proteins were characterized by their amino acid composition and starch-gel electrophoretic patterns. Comparisons were made with proteins obtained from endosperm meal by extraction with different solvents.

The slowest-sedimenting protein zone contained typical albumins and globulins soluble in centrifugation buffer. A second zone contained a high-molecular-weight protein soluble only in alkali. The amino acid profile of this glutelin protein differed significantly from glutelin extracted from meal with alkali. The third zone contained particles microscopically identical to free protein bodies. The protein in these bodies was characterized as zein. The fourth and fastest-sedimenting zone was identified as protein bodies surrounded by a protein matrix. The matrix protein, which resembled glutelin protein in its solubility properties, differs significantly from the extracted glutelin in amino acid composition. The electrophoretic patterns of the reduced and alkylated proteins of zones II and IV, when in combination, resemble the electrophoretic patterns of reduced and alkylated glutelin obtained by direct extraction of endosperm meal.

Examination of thin sections of normal corn endosperm under a light microscope reveals a protein network in the cells which not only holds together the starch granules but also contains spherical bodies averaging 2 μ in diameter (1). The proteinaceous character of both spherical bodies and matrix material was demonstrated by histochemical staining and proteolytic enzyme digestion (2). The protein bodies may contain zein, since treatment of the endosperm sections with 80% ethanol dissolved these bodies (2).

The importance of protein bodies as sites of deposition of storage proteins has been demonstrated in other seeds. Dieckert et al. (3) isolated subcellular fractions containing storage proteins of peanut cotyledon by zonal centrifugation. Tombs (4) studied the protein composition of protein bodies in cotyledons of soybeans.

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Australian workers (5) identified by microscopy protein bodies in developing wheat endosperm. The bodies from wheat contained acetic acid-soluble prolamines. All these studies indicate that specific proteins are located in definite structural components of the cell and that isolation of these subcellular particulates can serve as sources of specific proteins.

Since the time of Osborne (6), proteins have been isolated for characterization by sequential solvent extraction from defatted endosperm meals. An alternate approach to protein isolation, the isolation of subcellular particulates, was undertaken in this study to simplify the source of protein and minimize the interaction of protein with other proteins or with other constituents in the endosperm during extraction. Current interest in the genetic control of synthesis of different types and amounts of proteins within the seed has focused attention on the sites of protein accumulation. This paper describes the use of zone sedimentation as a means for isolating and purifying protein bodies, matrix proteins, and soluble proteins from yellow dent corn endosperm tissue. Evidence for the composition of proteins contained in particulates was based on microscopic and chemical analyses.

MATERIALS AND METHODS

Isolation of Particulate and Soluble Components from Endosperm

Endosperm was obtained from a normal yellow dent hybrid corn (Kelly No. 374) grown in Illinois during 1967 under field conditions and harvested at 24 days after hand pollination. Husked ears were immediately frozen and grain was removed from he cob in the field. Whole kernels were stored frozen. Endosperm was separated from the frozen kernel by hand dissection immediately before homogenization. In a typical density gradient run, endosperm pieces (3.2 g. dry weight) were homogenized for 10 min. in a Potter-Elvehjem homogenizer with 16.0 ml. of 0.1*M* potassium phosphate buffer (pH 7.5) containing 0.006*M* magnesium chloride. This buffer was approximately isotonic relative to the corn tissue. All manipulations of the endosperm tissue were performed at 2°C. to avoid bacterial contamination and minimize enzyme action. After homogenization, cell wall debris was removed by filtering through nylon bolting cloth (Swiss Nitex No. 153, 150 μ average size opening). Most of the starch granules were removed by allowing the homogenate (final volume 25 ml.) to stand for 3 hr. at 2°C.

The clarified homogenate (5 ml.) was immediately layered on the surface of 25 ml. of a 15 to 70% (w./v.) linear sucrose gradient, which had been prepared previously in 30-ml. centrifuge tubes. Sucrose solutions were prepared in pH 7.5 potassium phosphate buffer. Linear gradients were developed from equal volumes of 15 and 70% sucrose by means of a Phoenix Varigrad. Solutions were pumped from the Varigrad at 1.2 ml. per min. into the centrifuge tube. The gradients were cooled to 4°C. before the homogenate was layered on top of the tube. The tubes were then centrifuged at 4°C. in a swinging bucket rotor (Spinco SW 25.1) at 3,000 $\times g$ (4,500 r.p.m.) for 30 min. in a Spinco L-4 ultracentrifuge.

After centrifugation, zones of the different particulate components were detected visually and photographed. For this purpose tubes were illuminated from below with a concentrated beam of light and viewed against a dark background. Regions in the tubes containing suspended insoluble matter scattered light from its path, giving rise to bright zones.

Sequential fractions of 2.5-ml. volume were then removed from the top of the gradient solution through a hypodermic needle at a constant rate of 2 ml. per min. by means of a peristaltic pump. The needle was lowered 2 mm. into the solution for each fraction removed. This method caused very little mixing of the layers. The light-scattering zone in highest concentration adhered to the sides of the tube. When this material was reached, it was gently dispersed with a stirring rod into the top of the solution, and withdrawal of fractions was continued.

Suspended particulate components were removed from each fraction by centrifugation. To facilitate sedimentation of the particulates, 2 ml. of buffer was added to each fraction to reduce the density of the sucrose solutions. Centrifugation was conducted in polycarbonate tubes at 105,000 × *g* (30,000 r.p.m.) for 30 min. at 4°C. with type 30 rotor using a Spinco L-4 ultracentrifuge. Precipitates from all fractions were resuspended once with buffer and recentrifuged to remove any remaining sucrose. The supernatants from each fraction were dialyzed against water to remove sucrose and buffer and freeze-dried before analysis for soluble proteins. To obtain adequate amounts of samples for analytical studies, several runs were made and corresponding fractions combined. Reproducibility of the gradient and zone sedimentation was excellent as judged by measuring the positions of light-scattering zones within different tubes. The amount of alpha-amino nitrogen in the peaks from the separate fractions of several runs agreed within 2%.

Microscopy

Bright-field and phase microscopy were used to examine particulates for composition and purity. Contaminating starch was detected by staining with a dilute solution of I_2-KI.

Compositional Analysis

Relative amounts of protein in both the precipitate and supernatant portions of each of the fractions were determined by analysis for alpha-amino nitrogen with a Technicon Autoanalyzer. Aliquots of samples were dispersed in buffer and introduced into the instrument at intervals. They were then automatically hydrolyzed in 10% sodium hydroxide for 1 hr. at 94°C. and reacted with ninhydrin. Some of the isolates were extracted with different solvents to separate specific proteins before amino acid and electrophoretic analysis. Extraction procedures used were essentially those of Boundy et al. (7). Nitrogen was determined on protein isolates by the semimicro-Kjeldahl procedure.

Amino acid composition of hydrolyzed proteins was determined with an accelerated Phoenix amino acid analyzer and calculated on an IBM Model 1130 computer by means of a program developed by Cavins and Friedman (8). Material was refluxed in a large volume on 6*N* HCl for 24 hr. to prepare hydrolysates for analyses of amino acids.

Starch-Gel Electrophoresis

Samples for starch-gel electrophoresis were dissolved in aluminum lactate buffer (pH 3.5) to give a 5% protein concentration. Electrophoresis was carried out in starch gels prepared in this buffer by the method of Turner et al. (9) as modified by Beckwith et al. (10). For comparison, proteins were isolated by extraction of defatted mature yellow dent corn endosperm meal (7) and subjected to gel electrophoresis. Disulfide bonds of high-molecular-weight proteins were cleaved to

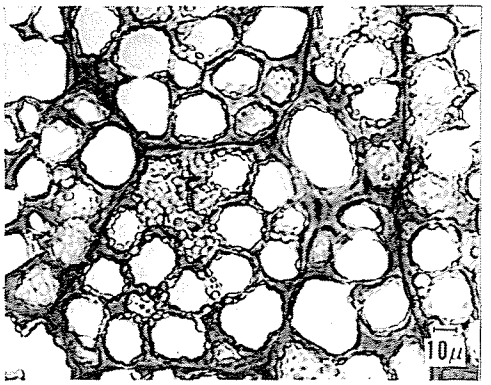


Fig. 1. Tangential section of mature endosperm corn tissue, cut dry with a glass knife, 3 μ thick, destarched with amylase, and stained with 0.1% I_2-KI solution.

test the electrophoretic behavior of the resulting polypeptides. The reduction of disulfides in mercaptoethanol and alkylation of the resulting sulfhydryls with acrylonitrile was carried out in phosphate buffer (pH 8.0, ionic strength 0.02) containing 8*M* urea. The solutions containing 20 to 100 mg. protein were stirred for 4 hr. after addition of 100:1 molar ratio of mercaptoethanol to all protein disulfide bonds. Acrylonitrile was then added in an equimolar ratio to the mercaptoethanol and reacted for 1 hr. at room temperature (11). The solutions were adjusted to pH 3.0, dialyzed for 48 hr. against 0.01*N* acetic acid, and lyophilized.

RESULTS AND DISCUSSION

Structure of Corn Endosperm

The photomicrograph in Fig. 1 shows the organization of mature corn endosperm cells. Starch granules originally in the large white spaces were enzymatically digested from the tissue with amylase to demonstrate more effectively the protein network and cell wall structure (2). Protein bodies, the small round structures within the cell, are associated with an amorphous matrix of protein. As the grain develops and the endosperm cells dehydrate, the protein bodies and starch granules enlarge, pack together, and become tightly bound to the protein matrix. Thus disruption of starch granules from other subcellular structures in mature endosperm requires vigorous conditions of steeping with reducing agents. In contrast, the immature cell particulates are more loosely associated, a condition that renders separation more feasible.

Homogenization and Starch Sedimentation

The 24-day endosperm tissue (milky stage) was readily homogenized in the buffered isotonic solution. Cell division is complete at this stage of kernel development and the proteins present are representative of those in mature cells, although their relative amounts differ in mature grain (12). Particulate structures like those in mature cells are also found at this stage of kernel development.

Cell debris and starch granules must be removed from the homogenate before density gradient separation of the protein-rich subcellular particulates. Cell debris was removed by filtering the homogenate through bolting cloth. Next, approximately 80% of the solids, primarily starch granules, was settled-out under

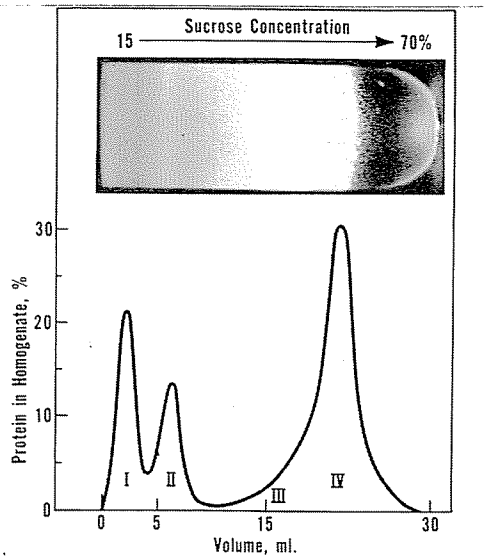


Fig. 2. Distribution of proteins in a homogenate of corn endosperm subjected to zonal centrifugation as determined by light-scattering (upper photo) and analysis of total α-amino nitrogen content of fractions (lower graph).

gravity. Ninety percent of the nitrogren of the endosperm was left dispersed in the clarified homogenate. The 10% nitrogen lost with the starch sediment was accounted for as protein bodies enveloped in a matrix protein. These bodies were microscopically observed on the surface of the solids that settled on standing. Variables of time and concentration during settling influenced the amount of nitrogen deposited in this sediment. The conditions used in these experiments are the most practical for starch removal with least nitrogen loss.

Density Gradient Centrifugation

Preliminary attempts to separate particulates from corn endosperm homogenate with a 15 to 40% sucrose gradient, as employed for wheat preparations by Graham et al. (5), were not successful. Only 55% of the total nitrogen of the corn homogenate was distributed through that gradient system; the remaining nitrogen was sedimented to the bottom of the tube. The 15 to 70% sucrose gradient used in most subsequent experiments resulted in almost complete suspension of the homogenate nitrogen in the gradient solution.

Figure 2 illustrates a typical view of the centrifuge tube after density gradient centrifugation, showing bright areas of scattered light due to suspended particulates from the endosperm homogenate. Protein concentrations were assessed by alpha-amino nitrogen analysis of alkaline-hydrolyzed fractions. Figure 2 also shows a plot of protein concentration *vs.* position in the tube. Three peaks were located. Two of these protein-rich regions correspond directly to the light-scattering zones within the tube. An uppermost zone (I) contained soluble albumins and globulins which did not exhibit light-scattering. Zone II contained an insoluble protein which scattered light. The protein-containing peak sedimenting most rapidly was skewed

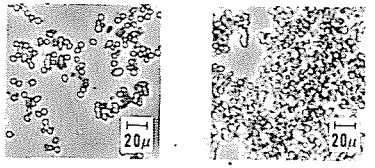


Fig. 3. Light-phase micrographs of free protein bodies (zone III) and matrixed protein bodies (zone IV). Material was mounted in a 1:1 glycerol:water solution and stained with 0.1% I_2-KI solution.

on the ascending side enough to indicate the presence of two components. Heterogeneity of the peak was also apparent from the light-scattering pattern. Therefore, this peak was divided into two zones, III and IV. Further attempts to effect better separation of these zones by gradient centrifugation with buffer solutions containing either ionic or nonionic detergents were not successful.

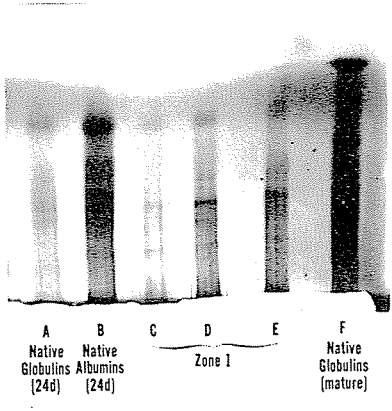
Microscopic Examination of Zones

Insoluble components of each of the zones were collected by ultracentrifugation and examined directly with a phase microscope. No particulates were observed in zones I and II. However, smaller structures may require magnification obtained with the electron microscope for recognition. Particulates were detected in zones III and IV by phase microscopy (Fig. 3). These particulates were not contaminated with starch granules or cell-wall material. Photomicrographs in Fig. 3 clearly show free protein bodies in zone III on the left and matrixed protein bodies in zone IV on the right. The spherical bodies from zone III are recognizable as free protein bodies by their size, shape, and structure. The spherical bodies are less distinct in the photomicrograph on the right where they are larger and clumped together with an amorphous substance. The larger size and greater association with matrix protein indicate that these bodies are at a more advanced stage of development than the free protein bodies. The excellent separations achieved permit further chemical characterization of the proteins contained in these particulates.

Analysis of Zones

Proteins contained in the particulates were characterized by their amino acid composition and their electrophoretic patterns on starch gels. Comparisons were made with proteins sequentially extracted with water, 0.5*M* sodium chloride, 70% ethanol, and 0.1*N* sodium hydroxide from both immature and mature defatted endosperm meal.

Zone I contained water-soluble albumins and salt-soluble globulins as characterized by their solubility in the phosphate buffer. This zone was separated into three fractions (upper, 1; middle, 2; and lower, 3). The fractions were ultracentrifuged at 105,000 $\times g$ to remove contaminating yellow-colored substances. Starch-gel electrophoretic patterns of proteins contained in the supernatants of fractions 1, 2, and 3 are shown in Fig. 4. Most of the proteins in



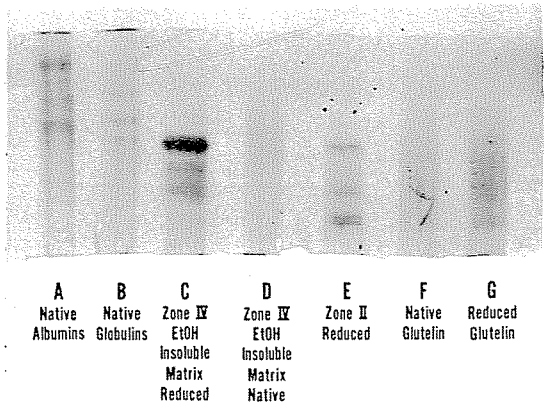


Fig. 4 (left). Comparison of starch-gel electrophoretic patterns of soluble protein components in zone I and albumins and globulins isolated by solvent extraction of defatted endosperm meal. (A) Immature globulin, 24-day Kelly No. 374; (B) immature albumins, 24-day Kelly No. 374; (C, D, E) upper, middle, and lower fractions of zone I; and (F) mature globulins, Funk G-83. Origin at bottom with migration toward cathode. Buffer system, aluminum lactate-8*M* urea, pH 3.5.

Fig. 5 (right). Starch-gel electrophoretic patterns of alkylated-reduced proteins isolated in zone II and alkylated-reduced matrix proteins from zone IV. (A) Native mature albumins, (B) native mature globulins, (C) alkylated-reduced ethanol-insoluble matrix proteins from zone IV, (D) native zone IV ethanol-insoluble matrix proteins, (E) alkylated-reduced zone II, (F) native mature glutelin, and (G) alkylated-reduced mature glutelin. Electrophoretic conditions as described in Fig. 4. Reference proteins were isolated directly from mature endosperm (Funk G-83) by solvent extraction.

zone I readily migrated into the gel with mobilities resembling those of albumins and globulins from both mature and immature endosperm. Differences occurred in amounts of the individual proteins in the fractions constituting the zone (patterns C, D, and E, Fig. 1).

The light-scattering protein located in zone II was not soluble in either 0.5*M* sodium chloride or 70% ethanol. This protein is, therefore, not albumin, globulin, or zein. It did dissolve in 0.1*N* sodium hydroxide as does glutelin. Its amino acid profile, however, differs significantly from that of glutelin extracted from the whole endosperm and is unique among cereal proteins (Table I). The isolated protein did not penetrate into the gel upon electrophoresis. However, after reduction of its disulfide bonds and alkylation of resulting sulfhydryls, four distinct polypeptides migrated electrophoretically into the gel in urea-aluminum lactate buffer. Mobilities of these polypeptides were similar to those of some of the polypeptides of reduced and alkylated glutelin from endosperm meal (Fig. 5).

Free protein bodies in zone III (13.8% nitrogen) contained zein, as indicated by solubility and amino acid analysis (Table I). The low level of lysine and the abundance of leucine, glutamic acid, and proline demonstrate that the major protein in the free protein bodies is zein. Differences in amino acid composition, when compared to that of zein extracted from endosperm, could be attributed to small amounts of nonzein proteins also found in the protein bodies. Further evidence for the predominance of zein was obtained by direct gel electrophoresis of

TABLE I. AMINO ACID COMPOSITION OF PROTEINS IN ISOLATED PARTICULATE COMPONENTS AND PROTEINS EXTRACTED FROM CORN ENDOSPERM (mM/16 g. N)

Amino Acid	Light-Scattering Protein Zone II	Free Protein Bodies Zone III	Matrixed Protein Bodies Zone IV		Extracted Corn Proteins (8)	
			Ethanol-Soluble	Ethanol-Insoluble	Zein	Glutelin
Lysine	51	5	1	22	1	17
Histidine	17	19	15	41	9	29
Ammonia	72	137	129	116	191	132
Arginine	48	16	13	30	11	29
Aspartic	80	36	37	31	47	41
Threonine	42	27	24	29	27	36
Serine	50	51	50	40	52	53
Glutamic	88	172	182	131	190	150
Proline	58	146	152	155	108	121
Glycine	70	29	19	50	19	61
Alanine	79	98	109	61	128	86
1/2 Cystine	.....	18	14	34	4	16
Valine	66	32	28	41	38	46
Methionine	21	16	13	14	3	16
Isoleucine	41	28	31	23	42	25
Leucine	72	144	175	85	178	99
Tyrosine	24	31	33	26	33	31
Phenylalanine	34	39	47	23	57	31

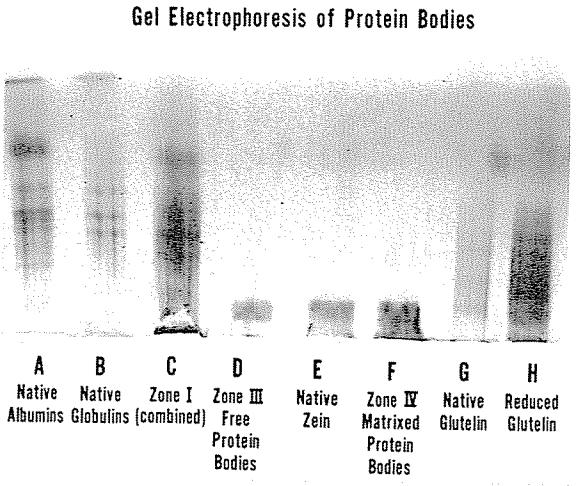


Fig. 6. Comparison of starch-gel electrophoretic patterns of protein components in free protein bodies of zone III and matrixed protein bodies of zone IV with zein extracted from mature Funk G-83 endosperm. Also, comparisons of mobilities of protein components in zone I (combined) with albumins and globulins extracted from mature Funk G-83 endosperm. (A) Native mature albumins; (B) native mature globulins; (C) zone I (combined); (D) zone III, free protein bodies; (E) native zein; (F) zone IV matrixed protein bodies; (G) native mature glutelin; and (H) alkylated-reduced mature glutelin. Electrophoretic conditions as described in Figs. 4 and 5.

solutions of zone III particulates in urea-aluminum lactate buffer (Fig. 6). The free protein bodies consisted primarily of proteins having low mobilities identical with those of alpha-zein (9).

Starch-gel electrophoresis of matrixed protein bodies of zone IV dispersed in urea-aluminum lactate buffer demonstrated the presence of both alpha- and beta-zeins (Fig. 6). The beta-zeins have high molecular weights and do not migrate far into the gel but appear as a streaked area near the origin (9). Separation of the zeins from the protein bodies in the matrixed material of zone IV was accomplished by alcohol extraction. More than 75% of the protein nitrogen in zone IV was soluble in 70% ethanol. The amino acid profile of the alcohol extract is similar to the amino acid composition of zein isolated directly from mature endosperm meal (Table I).

The matrix material (alcohol-insoluble residue from zone IV) was almost entirely protein (14.3% nitrogen). It was soluble only in 0.1*N* sodium hydroxide. Its amino acid composition differs significantly from that of glutelin (Table I). The matrix protein contains more lysine but less glutamic acid and leucine than does the glutelin extracted from endosperm meal. These amino acid differences indicate little or no zein component in the matrix protein. The gel electrophoretic pattern of reduced and alkylated matrix protein exhibited an intensely dark band and several minor bands comparable in mobilities to some of the bands in reduced and alkylated glutelin (Fig. 5). The slow moving pair of bands characteristic of zein in the urea-aluminum lactate buffer system is not present in the matrix protein.

CONCLUSIONS

These studies establish a relationship between structural components of the corn endosperm cell and the proteins classically defined by solubility. Protein bodies contain zein as characterized by solubility in 70% ethanol, amino acid composition, and starch-gel electrophoretic mobilities. Although earlier workers presented evidence for the location of zein in the protein bodies, this is the first time that these structures have been isolated and their proteins subjected to physical and chemical analyses. On the basis of analysis of other particulate structures, zein protein does not appear to be deposited in locations in the endosperm cell other than in the protein bodies. These studies provide conclusive evidence for Duvick's concept (1) of the protein body serving as the site of zein storage.

The matrix protein of zone IV and the protein in zone II have not previously been separated from corn endosperm tissue. Both of these proteins resemble glutelin because of their solubility in dilute alkali and insolubility in other solvents. Their amino acid compositions differ from that of glutelin that is obtained by alkali extraction of the endosperm meal. In the sequential extraction method some residual zein appears to be extracted with glutelin, which accounts for some of these compositional differences. Polypeptide chains of these proteins obtained after reduction and alkylation exhibit electrophoretic patterns similar to some of the bands within the pattern of the extracted glutelin. It would appear then that each of these proteins constitutes a part of the glutelin isolated by alkaline extraction from corn endosperm meal. Since the isolation method used here does not employ highly alkaline solvents, these native proteins are more representative of the

proteins contained in the endosperm cell than those obtained by the Osborne procedure (6). The sharp bands seen on the gel electrophoresis patterns indicate that only a few different polypeptide chains are involved in the formation of these proteins. Especially important is the fastest-moving electrophoretic component in the reduced and alkylated matrix protein in zone IV. The matrix appears to consist of polypeptide chains specific to this structurally important protein.

These techniques provide opportunity to study the relative amounts and types of glutelin proteins in new high-lysine genotypes of corn. Concentrations of glutelin proteins are increased genetically at the expense of zein and become more significant nutritionally in Opaque-2 and Floury-2 varieties (13). Isolation of these proteins under procedures that avoid denaturation and degradation provides opportunity for their direct analytical and nutritional evaluation. Since the protein bodies have been shown to be reduced in size in Opaque-2 (2), isolation of these bodies may provide more detailed information on their composition in this genotype. Further study of the subcellular structures should provide a better understanding of the synthesis and accumulation of storage proteins from different varieties of corn.

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