Subcellular Compartmentalization of Maize Storage Proteins in *Xenopus* Oocytes Injected with Zein Messenger RNAs

WILLIAM J. HURKMAN, L. D. SMITH, JOEL RICHTER, and BRIAN A. LARKINS

Department of Botany and Plant Pathology, and Department of Biology, Purdue University, West Lafayette, Indiana 47907

ABSTRACT   Maize storage proteins synthesized in oocytes were compartmentalized in membrane vesicles because they were resistant to hydrolysis by protease, unless detergent was present. The site of storage protein deposition within the oocyte was determined by subcellular fractionation. Optimal separation of oocyte membranes and organelles was obtained when EDTA and high concentrations of NaCl were included in the homogenization and gradient buffers. Under these conditions, fractions in sucrose gradients containing a heterogeneous mixture of smooth membranes (presumably endoplasmic reticulum, Golgi apparatus, and plasma membrane, density = 1.10–1.12 g/cm^3), mitochondria (densities = 1.14 and 1.16 g/cm^3), yolk platelets (density = 1.21 g/cm^3), and a dense matrix material (density = 1.22 g/cm^3) could be separated. Some zein proteins were recovered in the mixed membrane fraction, but the majority occurred in vesicles sedimenting with yolk platelets and granular material at a density of ~1.22 g/cm^3. When metrizamide was included in the gradient to increase the density, little of the dense matrix material was isolated, and vesicles containing zein proteins were separated from other oocyte components. These vesicles were similar to protein bodies in maize endosperm because they were of identical density and contained the same group of polypeptides.

The principal storage protein fraction in maize endosperm consists of a group of alcohol-soluble proteins called zein. During endosperm development, zein proteins are synthesized and deposited as protein bodies within the rough endoplasmic reticulum (1, 2). An analysis of zein proteins by SDS-polyacrylamide gel electrophoresis reveals two major components with 19,000 and 22,000 mol wt, as well as several minor components of 10,000 and 15,000 mol wt (3). However, isoelectric focusing of zein proteins resolves up to 28 polypeptides (4), indicating substantial charge heterogeneity among them.

Zein proteins are synthesized by membrane-bound polyribosomes (5), and when these polysomes or purified zein mRNAs are translated in vitro, the proteins synthesized are 2,000 mol wt larger than native zein polypeptides (2, 6, 7). In a previous study, we reported that when zein mRNAs were injected into *Xenopus* oocytes, zein proteins were synthesized with molecular weights identical to those of the native polypeptides (8). We also demonstrated that proteins from both sources had identical amino terminal sequences.

To determine whether the synthesis and processing of zein polypeptides in oocytes are accompanied by protein body formation, we have examined the subcellular distribution of zein proteins. We considered it important for these experiments to separate membranes directly from total oocyte homogenates by density gradient centrifugation. Because zein proteins form dense, insoluble deposits within rough endoplasmic reticulum (RER) membranes, they may have been lost or trapped among membranes if the oocytes were fractionated by differential centrifugation.

Previous research has shown that it is difficult to purify membranes from either total or partially fractionated oocyte homogenates (9, 10). Jared et al. (9) separated yolk platelets from mitochondria and a band of heterogeneous membranes. However, additional resolution of components in the membrane band was prevented by the appearance of precipitated or aggregated material. Zehavi-Wilner and Lane (10), in studies of the compartmentalization of secretory proteins in message-injected oocytes, fractionated oocytes by a procedure involving

two centrifugation steps. The first resolved a light and heavy membrane fraction when linear sucrose gradients were centrifuged for a short time at high gravitational force. Cellular components in both fractions were surrounded by a coarse matrix. A second step involving centrifugation with discontinuous gradients was necessary to separate cellular components from the matrix material, a procedure that was only partially successful.

We found that when high ionic strength buffers were used for homogenization of oocytes and density-gradient centrifugation, well-resolved membrane components could be obtained in a single centrifugation step. Membrane vesicles containing zein proteins were identified on the basis of their alcohol solubility and resistance to exogenous protease. Most of the zein proteins were localized in vesicles having the same density as protein bodies from maize endosperm.

MATERIALS AND METHODS

Preparation of Oocytes

Xenopus laevis obtained from South Africa or Nasco (Fort Atkinson, Wis.) were maintained as described by LaMarca et al. (11). Ovaries were removed from frogs anesthetized by hypothermia. Stage VI oocytes (12) were defolliculated manually and transferred to sterile Ringer's solution.

Oocytes were microinjected with 20 nl of zein mRNAs dissolved in sterile H_2O or OR_2 (13) at a concentration of 0.5 mg/ml. Zein mRNAs were purified by oligo(dT)-cellulose chromatography of membrane-bound polyribosomes from frozen kernels of the maize inbred line W64A, as previously described (2). The oocytes were incubated for ~24 h in OR_2, and then injected with 20 nl of [³H]leucine and incubated in fresh OR_2 for 4 h. The radioactive amino acid was prepared by adding 0.25 μmol of carrier leucine to 1 ml of 1 mCi/ml [³H]leucine (Amersham Corp., Arlington Heights, Ill.). The leucine was evaporated to dryness in a dessicator under vacuum and dissolved in 50 μl sterile H_2O. Optimal concentrations of mRNA and [³H]leucine, as well as incubation times and incubation media, were determined previously (8).

Cell Fractionation

50 oocytes were homogenized in 0.5 ml of buffer A (10% sucrose, 20 mM Tris-HCl [pH 7.6], 50 mM KCl, 10 mM $MgCl_2$) or buffer B (medium A plus 0.3 M NaCl and 2 mM EDTA) by passing them through a pasteur pipette several times. The unfiltered homogenate was layered onto an 11-ml, 20–60% (wt/vol) linear sucrose gradient, or a 10–50% linear metrizamide gradient. Sucrose gradient solutions were prepared in buffer A or buffer B in minus sucrose; metrizamide gradients were prepared in Buffer B containing 15% sucrose.

After centrifugation in the Beckman SW41 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 200,000 g for 2 or 18 h at 4°C, sucrose gradients were scanned continuously at 254 nm and fractionated with an ISCO model 640 gradient fractionator (ISCO[Instrumentation Specialties Co.], Lincoln, Nebr.). The density of gradient fractions was determined with an Abbe-3L refractometer (Bausch & Lomb, Inc. Scientific Optical Products Div., Rochester, N. Y.). Alternatively, membrane bands were visualized with a beam of light and collected with a pipette.

Total membranes of oocytes were obtained as follows: 50 oocytes were disrupted in buffer B containing 15% sucrose, and the homogenate was layered onto a 4.5-ml discontinuous gradient consisting of 1 ml of 2 M and 3.5 ml of 0.43 M sucrose in buffer B. After centrifugation in a Beckman SW50.1 rotor at 200,000 g for 1.5 h at 4°C, membranes were collected from the 0.43/2 M sucrose interface.

Enzyme Determinations

Rotenone-insensitive NADH cytochrome (Cyt) c reductase was assayed at 25°C by monitoring the reduction of Cyt c at 550 nm. The 3-ml reaction mixture contained 0.1 ml of the gradient fraction, 1.66 mM sodium cyanide, 120 μm Cyt c, 50 mM phosphate buffer (pH 7.5), and 230 μM NADH. The rate of Cyt c reduction was estimated with an extinction coefficient for Cyt c of 18.5 mM⁻¹ cm⁻¹ (14).

Cyt c oxidase was assayed by following the oxidation of Cyt c at 550 nm. Each 3-ml reaction volume contained 0.1 ml of the gradient fraction, 60 μM Cyt c reduced with Na-dithionite, 0.01% digitonin, and 50 mM phosphate buffer (pH 7.5). The rate of Cyt c oxidation was calculated according to Smith (15). Galactosyl transferase, glucose-6-phosphatase, and 5′-nucleotidase were assayed

as described by Morré (16); Na⁺-stimulated K⁺-ATPase was determined by the procedure of Wallach and Kamot (17).

Treatment of Membrane Fractions

Each membrane fraction was divided equally into three samples: to the first, no addition was made; to the second, 150 μg/ml protease K was added; and to the third was added protease K plus 0.1% SDS. Samples one and two were incubated at 4°C for 30 min to maintain membrane integrity, whereas sample three was incubated at 25°C for 30 min. Following incubation, the samples were made 250 μg/ml with phenylmethylsulfonyl fluoride (PMSF), an inhibitor of protease K. Membrane fractions from sucrose gradients were then extracted with 70% ethanol, containing 250 μg/ml PMSF, to solubilize zein proteins. Sucrose was removed by dialysis against 70% ethanol, and the samples were lyophilized for analysis. Membrane fractions from metrizamide gradients were recovered by centrifugation at 200,000 g in a Beckman SW41 rotor after the samples were diluted with at least 4 vol of the gradient buffer containing 15% sucrose.

Analysis of Labeled Proteins

Zein proteins were extracted at 60°C for 15 min in 70% ethanol; insoluble proteins were removed by centrifugation at 10,000 g in a Sorvall SS-34 rotor (DuPont Instruments-Sorvall, Dupont Co., Newtown, Conn.). Zein proteins were analyzed by SDS-polyacrylamide gel electrophoresis, as previously described (2). Lyophilized zein samples were dissolved in sample buffer (24 mM Tris-HCl, pH 8.3, 1% SDS, 1% 2-mercaptoethanol, 0.002% bromophenol blue, 10% glycerol), after first being heated in a boiling water bath for 3 min, and then applied to the gel. Zein precursor proteins were obtained by translation of zein mRNAs in a cell-free protein synthesis system prepared from wheat embryos (2). Radioactivity in gels was detected by fluorography, using preexposed Kodak RP X-Omat film (18).

Native zein proteins, and zein proteins synthesized in oocytes, were also analyzed by two-dimensional gel electrophoresis as described by O'Farrell et al. (19). The first-dimension electrofocusing gels were cast in 11.5 cm × 0.25 cm tubes, and contained 4% acrylamide, 9 M urea, pH 2–11 mixed ampholytes, and 1.6% Nonidet P-40 (NP-40). Samples were dissolved in 20 μl of lysis buffer (9.5 M urea, 2% NP-40, 2% ampholytes, pH 2–11, and 5% 2-mercaptoethanol). Samples were focused from anode to cathode for 1,800 V-h. Following electrofocusing, the gels were extracted from the tubes and equilibrated in an SDS buffer containing 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 25 mM Tris-HCl, pH 6.8. One gel was sliced into 4-mm sections that were soaked for 1 h in 1 ml of degassed, distilled H_2O to determine the pH gradient. The first-dimension gel was mounted onto a second-dimension 12.5% SDS polyacrylamide gel. After electrophoresis, gels either were stained with Coomassie Blue or analyzed by fluorography (18).

Electron Microscopy

Oocyte cell fractions were fixed with 0.5% glutaraldehyde for 1 h at 4°C; aqueous 10% glutaraldehyde was added directly to gradient fractions. The membrane fractions were then diluted five-fold with buffer B and centrifuged at 200,000 g for 1 h. The pellets were rinsed with buffer B and postfixed in the dark for 2 h in buffered 2% osmium tetroxide. Fixed specimens were rinsed in water, soaked 8–12 h in 0.5% uranyl acetate, dehydrated in acetone, and embedded in Epon. Thin sections were stained with lead citrate and viewed with a Philips EM200 electron microscope.

RESULTS

To determine whether synthesis and processing of zein precursor polypeptides was accompanied by compartmentalization within membrane vesicles, we isolated total oocyte membranes by discontinuous gradient centrifugation. Zein proteins sequestered inside membrane vesicles were distinguished from those nonspecifically associated by treating samples with protease in the presence and absence of detergent. In our initial experiments, we used a combination of trypsin and chymotrypsin, but, because of their amino acid composition (3), the zein proteins were not readily hydrolyzed by these enzymes. However, protease K was found to be very effective, even when SDS was used to disrupt membranes.

The stability of zein proteins in a total membrane preparation is illustrated in Fig. 1. Most of the labeled proteins
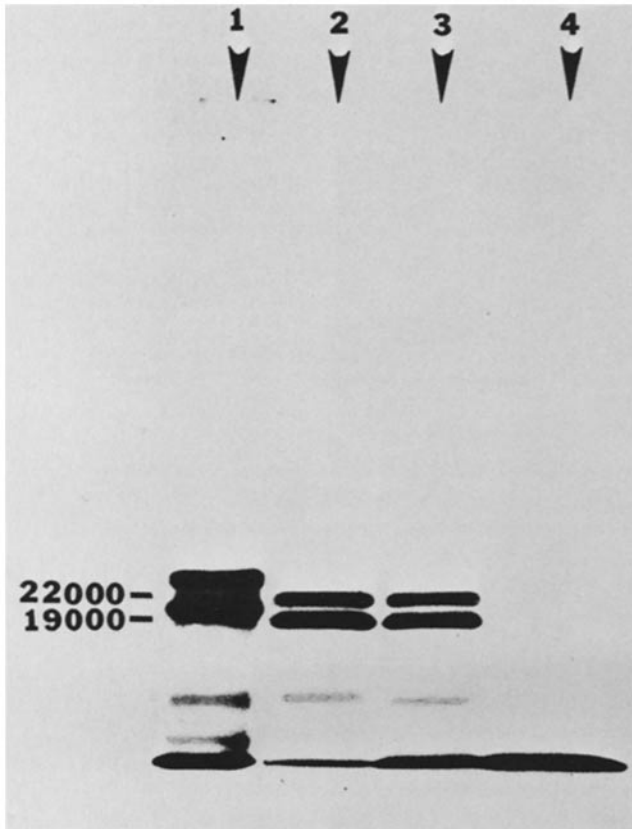
FIGURE 1   SDS-polyacrylamide gel analysis of ethanol-soluble proteins from the total membrane fraction of oocytes injected with zein mRNAs and [³H]leucine. Lane 1, zein mRNA translation products from a wheat germ cell-free system. Lane 2, membrane fraction extracted directly with ethanol. Lane 3, membrane fraction extracted with ethanol after incubation in 150 µg/ml protease K. Lane 4, membrane fraction extracted with ethanol after incubation in protease K and 0.1% SDS.

extracted with 70% ethanol from intact membrane vesicles (Fig. 1, lane 2) were resistant to protease K treatment (Fig. 1, lanes 2 and 3). However, when detergent was added (Fig. 1, lane 4), nearly all of the proteins were degraded to small molecular weight polypeptides. This experiment demonstrated that the zein proteins were compartmentalized within membrane vesicles; to determine the nature of these vesicles, we fractionated the oocyte homogenate by sucrose gradient centrifugation.

Fractionation of oocyte extracts by means of linear sucrose gradient centrifugation has previously been performed with buffers of low ionic strength and short centrifugation times (9). Therefore, in our initial experiments, oocyte homogenates were analyzed in sucrose gradients containing buffer A, and centrifuged for 2 h. Gradient fractions were monitored for activities of the following enzyme markers: NADH Cyt c reductase (endoplasmic reticulum [ER], mitochondria), NADPH Cyt c reductase (ER, mitochondria), glucose-6-phosphatase (ER), 5' nucleotidase (plasma membrane), galactosyl transferase (Golgi apparatus), and Cyt c oxidase (mitochondria). Except for NADH Cyt c reductase and NADH Cyt c oxidase, all other enzyme activities were below the detectable limits of the assays. Similar results were reported by Zehavi-Wilner and Lane (10).

After a 2-h centrifugation, ER and mitochondria sedimented at densities of 1.17 and 1.21 g/cm³, respectively, which was apparently due to association with yolk platelets. Aggregation of yolk platelets is reduced by EDTA, and yolk platelet protein

(vitellogenin) is soluble in high concentrations of NaCl (20). Therefore, in subsequent experiments, EDTA and NaCl were included in the buffers.

Addition of EDTA and NaCl to the homogenizing medium and gradient buffer resulted in far better separation of cellular components. In addition to a lipid layer at the top of the gradient, there were four peaks of UV-absorbing material sedimenting at densities of 1.10, 1.14, 1.16, and 1.20 g/cm³ (Fig. 2). Some dense viscous material, as well as pigment granules, sedimented to the bottom of the gradient. The dense viscous material did not pellet when a 2-M sucrose cushion was included in the gradient (Fig. 3a), and it was subsequently found to have a density of 1.22 g/cm³.

Cyt c reductase activity was found in all four of the UV-absorbing peaks, although it was most prominent in bands 2 and 3. Because Cyt c oxidase activity was also associated with these two peaks, they appeared to be mitochondria.

Electron microscopy of the gradient fractions revealed that band 1 (Fig. 3b) consisted of a heterogeneous mixture of membrane vesicles, bands 2 and 3 (Figs. 3c, d) of mitochondria, and band 4 of yolk platelets (Fig. 3e). Band 5 contained amorphous material (Fig. 3f), which may be equivalent to the "precipitated or aggregated material" and "granular matrix" observed by Jared et al. (9) and Zehavi-Wilner and Lane (10).

Because this fractionation procedure yielded well-resolved subcellular components, we used it to monitor the distribution of zein proteins in identical fractions from mRNA-injected oocytes. To distinguish zein proteins that were within vesicles from those simply adsorbed onto membranes, we again compared fractions treated with protease and protease plus detergent with those extracted directly with alcohol. Some zein
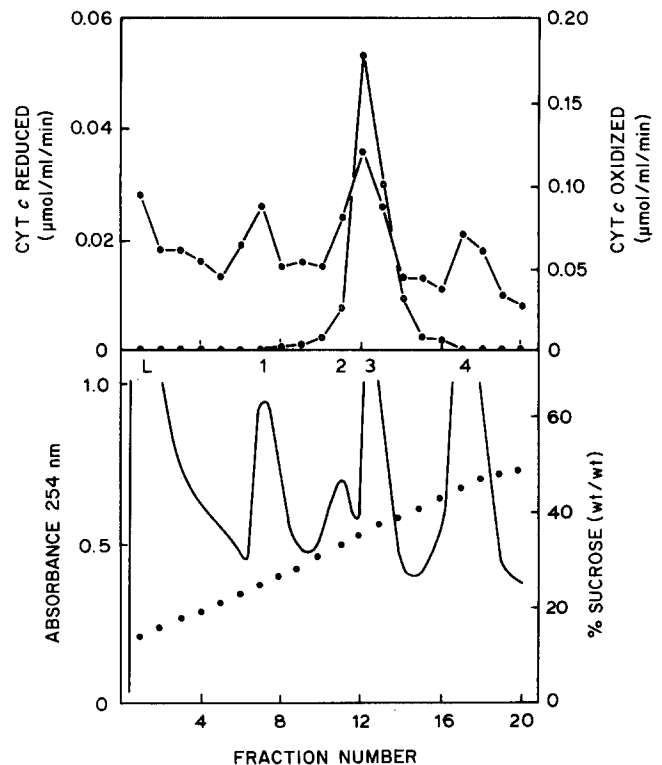

FIGURE 2   Sucrose density gradient analysis of total oocyte homogenate in buffer containing 2 mM EDTA and 0.3 M NaCl. The gradient was centrifuged for 18 h at 200,000 g in a Beckman SW41 rotor. Marker enzymes: O, Cyt c oxidase; —●—, Cyt c reductase; ●, % sucrose; ——, UV absorbance.
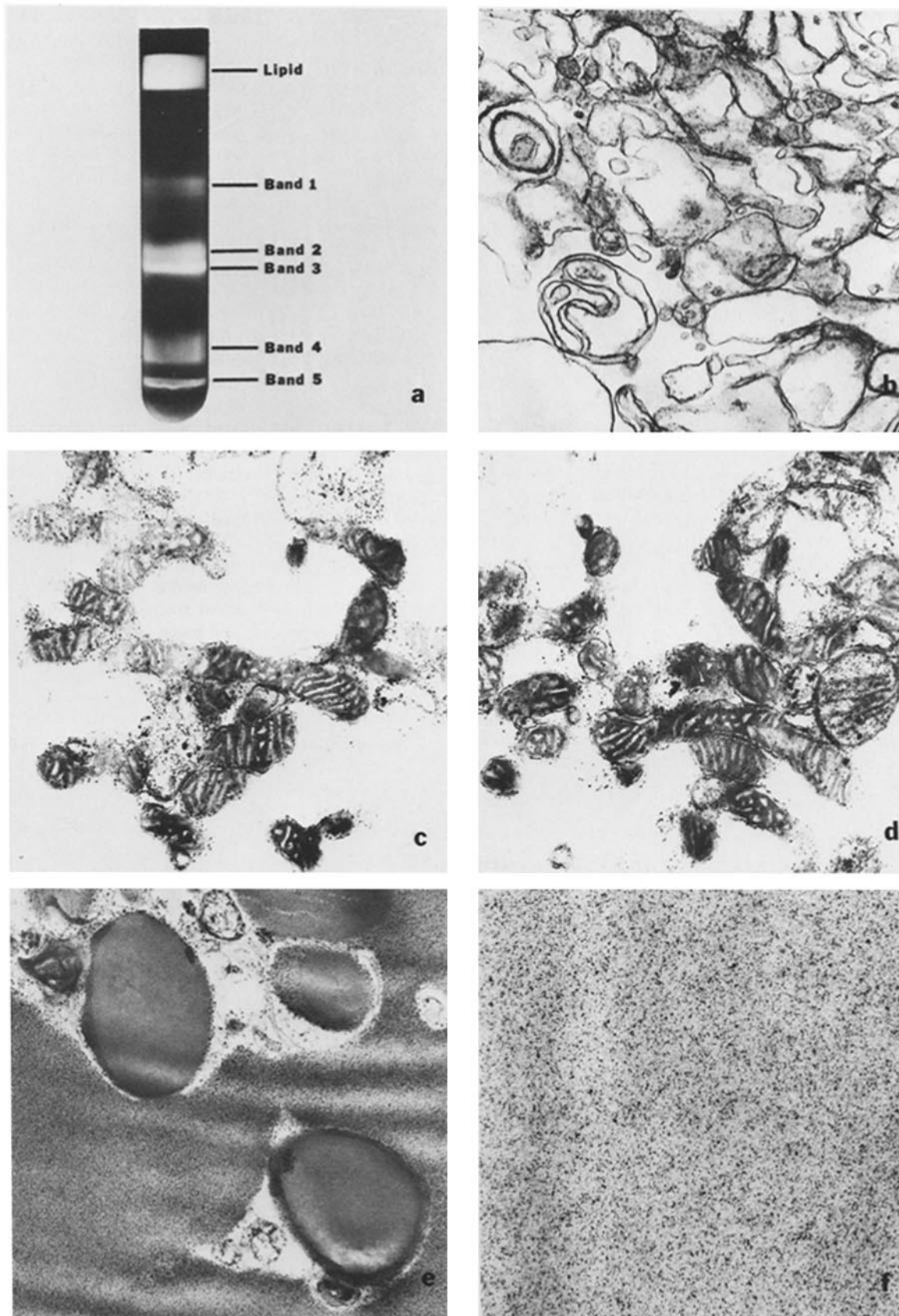
FIGURE 3   Electron micrographs of oocyte subcellular fractions. Fractions were collected from a 20–60% sucrose gradient (see Fig. 2). (A) Distribution of membrane bands visualized by light scattering. (B) Band 1, heterogeneous mixture of membrane vesicles (× 38,200). (C) Band 2, mitochondria, (× 37,700). (D) Band 3, mitochondria (× 37,700). (E) Band 4, yolk platelets (× 27,600). (F) Band 5, dense granular material × 38,200.

proteins were associated with each of the membrane fractions. However, much of this protein was susceptible to hydrolysis by protease, indicating that it was nonspecifically adsorbed onto membrane surfaces. Zein proteins that were resistant to hydrolysis by protease (Fig. 4, lanes *1B*, *4B*, and *5B*) were susceptible to protease in the presence of detergent (Fig. 4, lanes *1C*, *4C*, and *5C*). Most of this protein was within membrane vesicles that banded at a density near 1.22 g/cm³, where protein bodies from maize endosperm sedimented in the gradient. However, because these vesicles cosedimented with yolk platelets and the dense matrix material, we could not rule out aggregation or trapping of vesicles containing zein proteins.

We were unable to overcome the problem of cosedimentation by using only sucrose gradients. However, we found that gradients of metrizamide effectively separated vesicles containing zein proteins from other oocyte components (Fig. 5 *a* and *b*). The composition of subcellular fractions resolved by metrizamide gradients was identical to that obtained with sucrose gradients, except that most of the dense matrix material was associated with the yolk platelet fraction. In addition, because of the increased density, the other membrane bands sedimented more closely together.

The distribution of zein proteins from mRNA-injected oocytes was analyzed by use of these gradients, and most of the zein proteins were recovered from the region of the gradient where protein bodies sedimented. Nearly all of the protein in this fraction was resistant to hydrolysis by protease, indicating that it was compartmentalized within membrane vesicles (Fig. 6).

As an additional criterion to establish the identity between zein proteins from oocyte and maize endosperm protein bodies, the proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis (19). Zein proteins from both sources showed similar patterns in these gels (Fig. 7). The 22,000-mol wt polypeptides were separated into five differently charged species, whereas the 19,000-mol wt component was separated into two major and several minor charged forms. There were

two different charged forms of the 15,000-mol wt polypeptides resolved in the oocyte sample, but only one in the endosperm preparation. Both samples showed trace amounts of a 10,000-mol wt polypeptide. Both samples also contained a higher molecular weight form (Fig. 7 "*a*") that did not appear in samples analyzed only on SDS polyacrylamide gels. The origin of this protein is unknown; it may be an aggregate of several of the smaller polypeptides.

DISCUSSION

Although the value of *Xenopus* oocytes for studying posttranslational modification of proteins has been widely recognized, it has been difficult sometimes to determine the subcellular localization of specific proteins. This problem appears to result from the association and aggregation of membrane components with yolk platelets during oocyte homogenization. We found that this association was substantially reduced if EDTA and high concentrations of NaCl were included in the grinding and gradient buffers. As a result, distinct membrane fractions could be separated using a single density gradient centrifugation.

Identification of these membranes by marker enzymes was hampered by low enzyme activity, and only the distribution of mitochondria (NADH Cyt *c* oxidase) and ER membranes (NADH Cyt *c* reductase) could be determined. However, by monitoring the distribution of these enzymes and analyzing membrane fractions by electron microscopy, we were able to determine the origin of most membrane bands.

The Cyt *c* reductase activity sedimenting at a density of 1.1 g/cm³ was associated with a heterogeneous mixture of smooth membranes. In addition to ER, this fraction would be expected to contain a mixture of endomembranes, including Golgi and plasma membranes. However, these must be present in oocytes in small enough quantities that they can not be enzymatically detected. Some of the Cyt *c* reductase activity remained associated with the yolk platelet fraction, even when EDTA and high concentrations of NaCl were included in the buffer. This
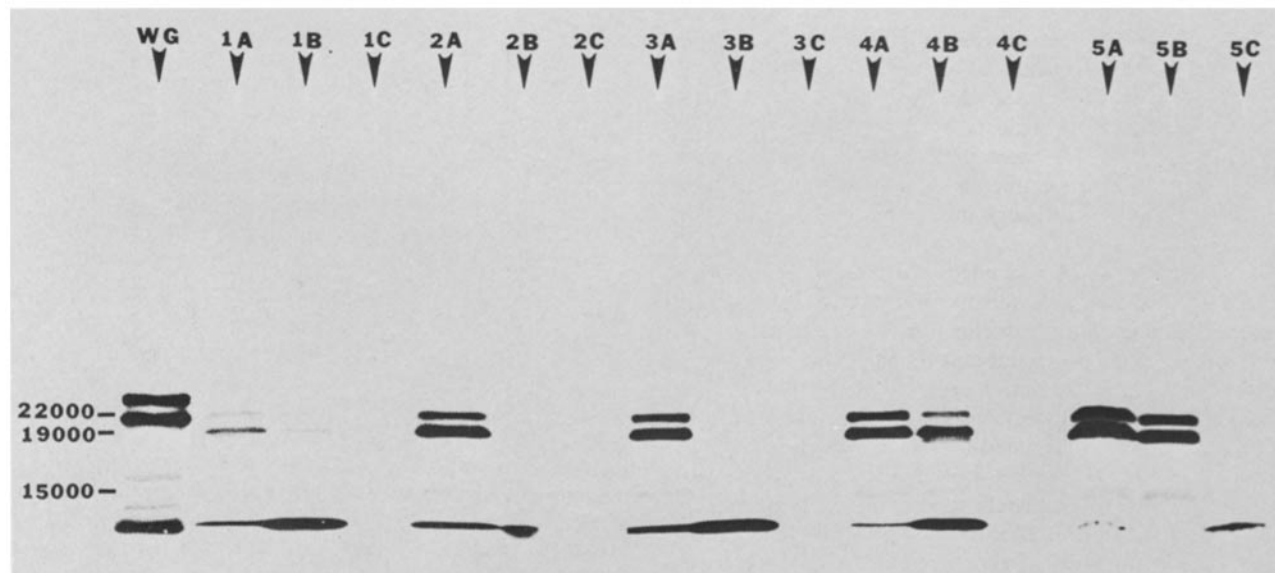

FIGURE 4   SDS-polyacrylamide gel analysis of ethanol-soluble proteins in membrane fractions isolated from oocytes injected with zein mRNAs and [³H]leucine. Numbers denote bands *1-5* in sucrose density gradients (see Fig. 3). Letters denote treatments of membrane fractions: *A*, membranes extracted directly with ethanol; *B*, membranes extracted with ethanol after incubation in 150 µg/ml protease K; *C*, membranes extracted with ethanol after incubation in protease K and 0.1% SDS. *WG*, sample from wheat germ cell-free translation system.
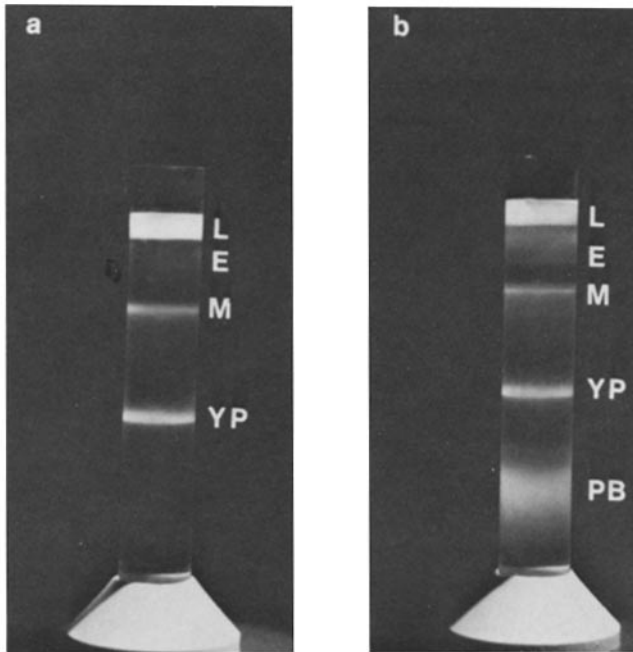
FIGURE 5 Analysis of *Xenopus* oocyte and maize endosperm extracts with metrizamide gradients. Oocyte homogenates and metrizamide gradients were prepared as described in Materials and Methods. Gradients were centrifuged for 18 h at 200,000 g in a Beckman SW41 rotor. The position of membrane bands was visualized by passing a beam of light through the gradient: (a) oocyte extract only; (b) oocyte extract plus endosperm homogenate. The positions of the lipid layer (L), endoplasmic reticulum (E), mitochondria (M), yolk platelets (YP), and protein bodies (PB) are indicated.

presumably reflects residual aggregation of cellular membranes with yolk platelets. Thus, although this procedure substantially improved the purification of endomembranes, it did not completely eliminate the aggregation problem.

We were unable to determine the origin of the dense matrix material that sedimented to the bottom of the sucrose gradient. Electron microscopy revealed that this material was nondescript, with occasional clumps of aggregated membranes and organelles. This material appeared to sediment with yolk platelets when metrizamide gradients were used, since a distinct layer did not appear in these gradients. We noticed more matrix material in electron micrographs of yolk platelets from metrizamide gradients, although the reason for this association is not apparent.

When extracts from oocytes injected with zein mRNAs were analyzed in linear sucrose gradients, zein proteins were present in several fractions. Some proteins were nonspecifically associated with each of the membrane bands. This most likely occurred during homogenization and resulted from the hydrophobic nature of the polypeptides. Indeed, when zein proteins synthesized in vitro were added to oocyte homogenates, they became associated with each membrane fraction. These proteins were, however, completely susceptible to hydrolysis by protease (data not shown). In mRNA-injected oocytes, a small amount of zein in the ER fraction was resistant to protease, but most of the protease-resistant protein sedimented with the fractions containing yolk platelets and the dense matrix material. Because endosperm protein bodies also sedimented to this region of the gradient (d 1.21–1.22 g/cm^3), this result was suggestive that zein proteins did associate to form protein

bodies inside oocyte ER vesicles. The small amount of zein sedimenting in the ER fraction probably resulted from protein that was insufficiently complexed.

More direct evidence for the formation of protein bodies inside oocytes was obtained by fractionation using metrizamide gradients. These gradients eliminated the band of dense matrix material and effectively separated vesicles containing zein proteins from other oocyte components. Protein bodies isolated from oocytes by this method appeared to be identical to those from maize endosperm, at least with respect to density and polypeptide composition. We analyzed samples from this region of the gradient by electron microscopy and found structures morphologically resembling protein bodies (C. E. Bracker, unpublished observations). However, localization of zein proteins will require autoradiographic analyses.

The zein proteins were found to be stably retained in these membrane vesicles. The majority of labeled storage protein could be recovered from oocytes several days after injection of radioactive amino acids, and we were never able to detect them outside the oocyte in the incubation medium. Therefore the posttranslational processing of maize storage proteins in oocytes differs somewhat from that of animal secretory proteins that have been shown to be secreted outside the cell (21). This may be a consequence of the storage proteins associating to form dense aggregates (protein bodies) within RER membranes.

Our results demonstrate that plant proteins can be synthesized, processed, and compartmentalized by oocytes injected with mRNA. Although it is likely that zein proteins accumulate within oocyte membranes by a translation-coupled transfer of nascent polypeptides, evidence presented here is not enough to exclude the possibility that prezein proteins synthesized and released from membrane-bound ribosomes are transferred across membranes where signal peptides are subsequently cleaved. However, it has been shown that posttranslational incubation of presecretory proteins with microsomal mem-
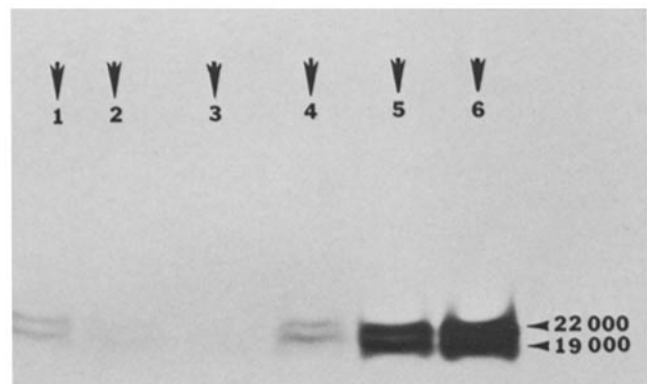

FIGURE 6 SDS-polyacrylamide gel analysis of ethanol-soluble proteins extracted from oocyte membranes separated by metrizamide gradient centrifugation. Gradient fractions were incubated in the presence or absence of 150 μg/ml protease K for 30 min at 4°C, then the metrizamide was diluted at least fourfold with grinding buffer, and the membranes pelleted by centrifugation at 200,000 g for 5 h. Membrane pellets were extracted with 70% ethanol, and the ethanol-soluble proteins were analyzed. Lanes 1 and 2: protein extracted from the mitochondria fraction before and after protease treatment; lanes 3 and 4: proteins from the yolk platelet fraction before and after protease treatment; lanes 5 and 6: proteins from the region of the gradient corresponding to the position of maize endosperm protein bodies.
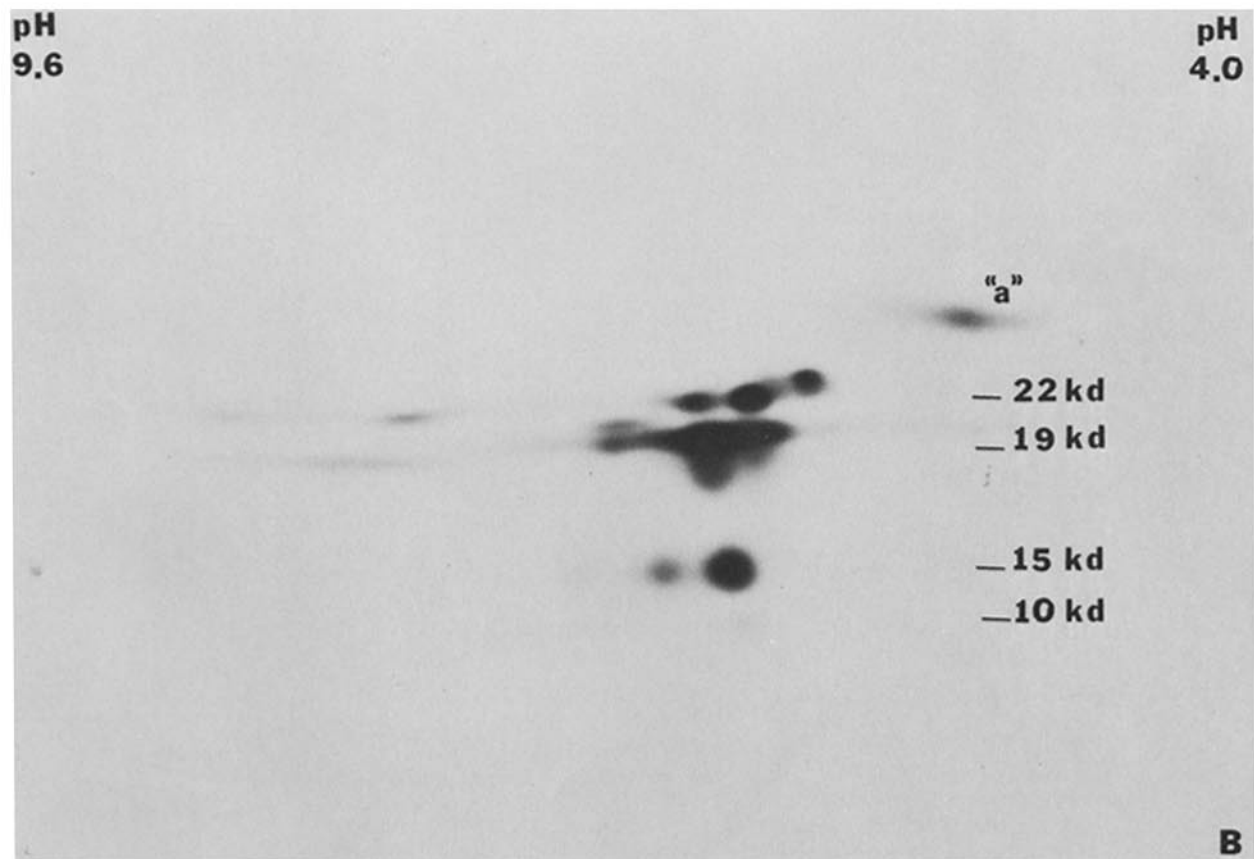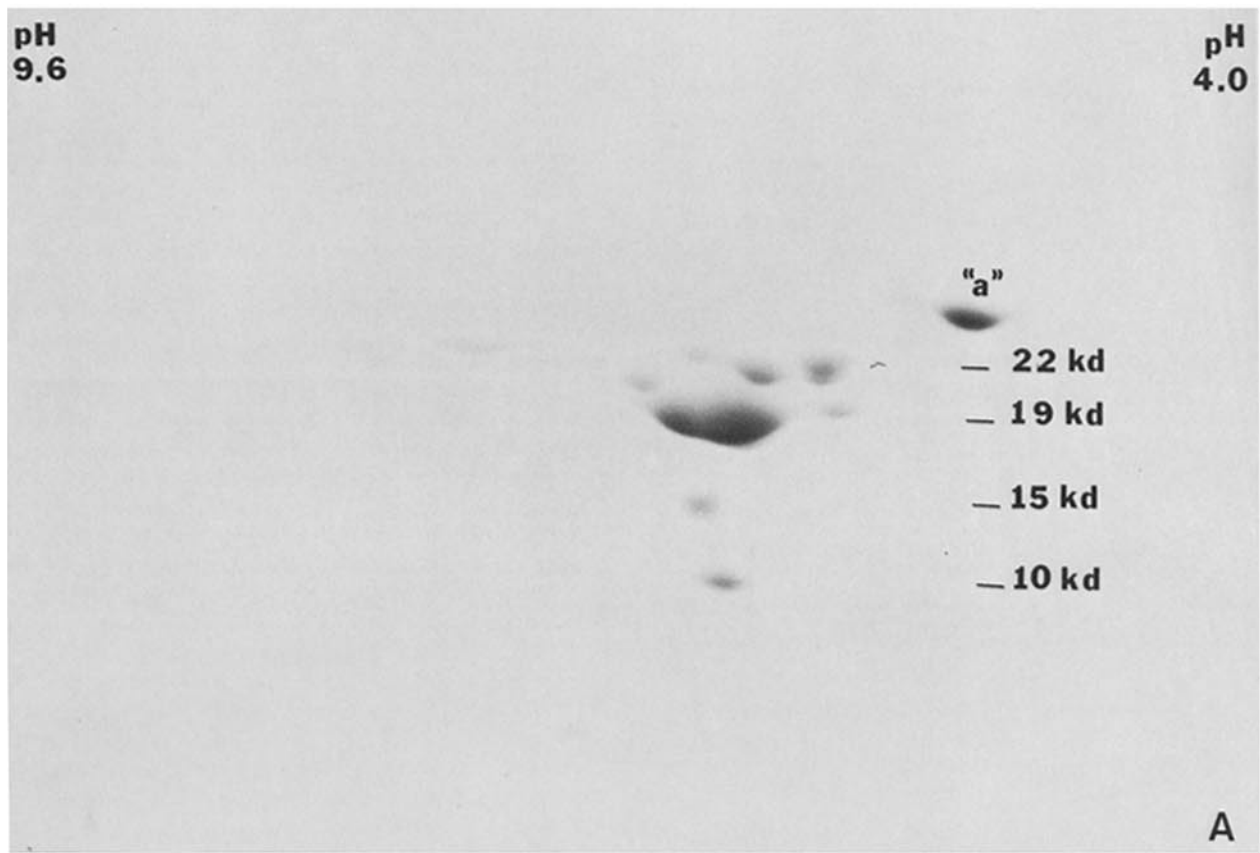
FIGURE 7   Two-dimensional polyacrylamide gel analysis of zein proteins from maize protein bodies (A) and from oocyte protein bodies (B). The pH gradient and molecular weights of polypeptides are indicated.

branes resulted in neither protein segregation nor removal of signal sequences (22, 23).

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