

Protein Bodies from the Endosperm of Castor Bean

SUBFRACTIONATION, PROTEIN COMPONENTS, LECTINS, AND CHANGES DURING GERMINATION¹

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ABSTRACT

Protein bodies from the storage endosperm of dry castor bean (*Ricinus communis* L.) were isolated by successive nonaqueous linear density gradient centrifugation. The isolated protein bodies were lysed by the addition of water, and the various structural components of the organelles were separated by sucrose gradient centrifugation. The matrix protein remained at the top of the gradient while the membrane, the crystalloids, and the globoids migrated to densities 1.15 g/cm³, 1.30 g/cm³, and > 1.46 g/cm³, respectively. The protein of the protein bodies was distributed evenly between the crystalloids and the matrix, and little protein was present in the globoids or the membrane.

The proteins of the protein bodies were resolved into protein components of diverse molecular weights in sodium dodecyl sulfate-acrylamide gel electrophoresis. The protein components of the organelle matrix were distinct from those of the crystalloids. Whereas the matrix proteins had very diverse molecular weights, the crystalloid proteins were mainly composed of several proteins with molecular weights between 50,000 and 60,000 daltons. Also, the matrix proteins were soluble in water while the crystalloid proteins were insoluble in water but soluble in salt solution, thus representing albumins and globulins, respectively. Two of the matrix proteins with molecular weights approximately 120,000 and 65,000 daltons were identified as the phytohemagglutinin and the toxic protein ricin, respectively.

During germination, the crystalloid proteins served as the storage protein and went through rapid degradation with smaller polypeptides formed as intermediates. In contrast, the proteins of the matrix underwent a much slower degradation during the same period and did not appear to be storage protein.

The storage proteins of many seeds are localized in spherical organelles called protein bodies (1, 5, 6, 12, 21, 23). The organelles are bounded by a single membrane with sizes ranging from 1 to over 10 μm in diameter depending on the plant species. Many hydrolytic enzymes that are active during germination are also present inside the organelles (2, 3, 7, 16). The protein bodies have an amorphous protein matrix, and in most legumes, the organelles also contain inclusions of phytin globoids (1). In some seeds, such as hempseed (19) and castor bean (17, 20), the protein bodies possess extra inclusions of protein crystalloids in addition to the phytin globoids. In hempseed, the isolated protein crystalloids contain edestin, the storage protein of the seed (17). In castor bean, the phytin is restricted to the globoids of the organelles (20). The functions and composition of the matrix proteins have not been studied, and the physiologi-

cal relationship between the matrix and the crystalloids is unknown.

In dry castor bean, there are two proteins of special interest: ricin, a toxic protein that strongly inhibits protein synthesis of eucaryotic cells, and a phytohemagglutinin, a lectin which agglutinates erythrocytes (9). They are both glycoproteins of mol wt 60 to 65,000 and 120,000 daltons, respectively. Even though they have been purified and studied intensively, their physiological roles and subcellular localization within the castor bean cell have not been investigated.

In this paper, we report the isolation of intact protein bodies from dry castor bean and the subfractionation of the organelles into the various structural components. We demonstrate that there is no overlapping in the protein constituents between the matrix and the crystalloids of the organelles. We also establish that the crystalloids are composed of storage protein of the globulin type, whereas the matrix contains nonstorage albumin proteins, including ricin and phytohemagglutinin. Similar investigation was carried out independently by Tully and Beevers; part of their work has been published in an abstract (22).

MATERIALS AND METHODS

Electron Microscopy. Endosperm of dry castor bean (*Ricinus communis* L. var. Hale) was cut into pieces of approximately 1 mm³ and fixed in 3.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, for 12 hr. The tissue was rinsed several times in the same buffer with sucrose added to maintain the same osmolality. The tissue was postfixed in 1% OsO₄ in 0.1 M Na phosphate buffer, pH 7.4, at the same osmolality for 2 hr, dehydrated with increasing concentrations of ethanol, and embedded in Spurr. Sections were stained in 5% uranyl acetate (24) for 20 min followed by lead citrate (18) for 5 min, and observed under a Zeiss EM9 electron microscope. A Zeiss phase contrast microscope was used for light microscopy.

Isolation and Subfractionation of Organelles. Protein bodies were isolated by successive nonaqueous linear density gradient centrifugation. Eight g of deshelled dry castor bean were ground in 20 ml of hexane at low speed in a VirTis 45 homogenizer for 5 min. The homogenate was filtered through a Nitex cloth of 35 μm^2 pore size. Two ml of the filtrate were layered onto a 32-ml linear gradient of hexane and carbon tetrachloride from density 1.20 g/cm³ to 1.50 g/cm³. The gradient was centrifuged at 21,000 rpm for 2 hr in a Beckman L2-65 B ultracentrifuge using a SW 27 rotor. After centrifugation, the gradient was fractionated and the fractions were observed under the light microscope. Most of the protein bodies were present at density around 1.36 g/cm³. The fractions with the protein bodies were pooled together and made to 1.50 g/cm³ with CCl₄. The preparation was then put in a centrifuge tube and a similar gradient of hexane-CCl₄ was layered upon it. Centrifugation was performed as

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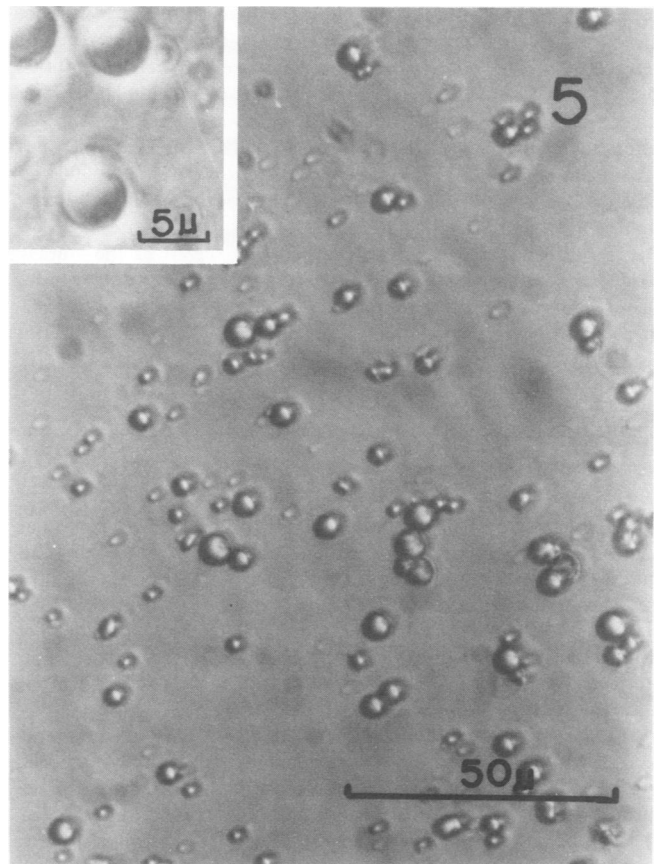
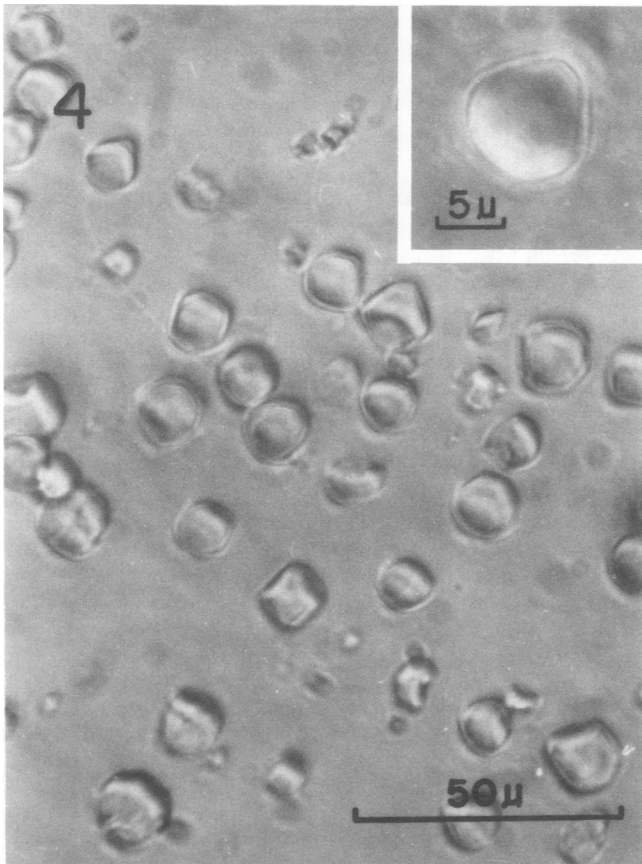
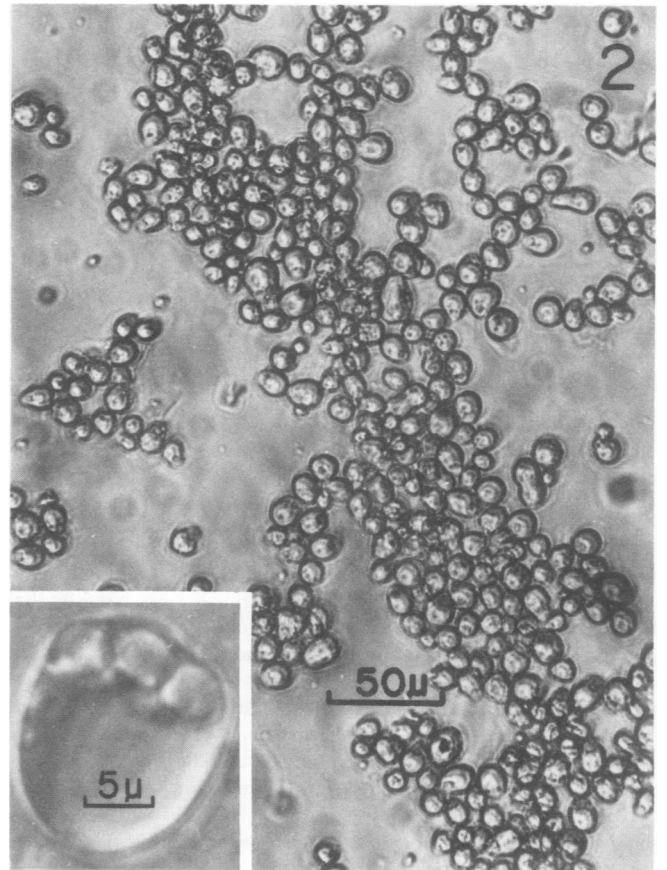
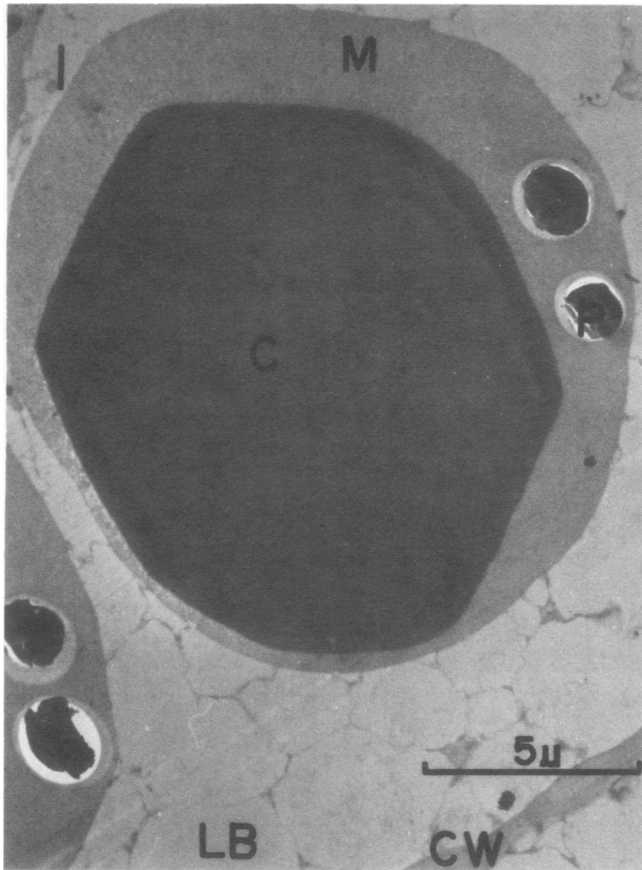


FIG. 1. Electron micrograph of a section of an endosperm cell of dry castor bean, showing a protein body with a large crystalloid (C), matrix (M), and two phytin globoids (P). A densely packed background of lipid bodies (LB) and part of a cell wall (CW) are also visible. $\times 6,000$.

FIG. 2. Light micrograph of protein bodies isolated by successive nonaqueous gradient centrifugation. $\times 400$. Inset shows an enlarged protein body with its inclusions.

FIG. 4. Light micrograph of the isolated protein crystalloids of the protein bodies. $\times 720$. Inset shows an enlarged crystalloid.

FIG. 5. Light micrograph of the isolated phytin globoids of the protein bodies. $\times 720$. Inset shows an enlarged globoid.

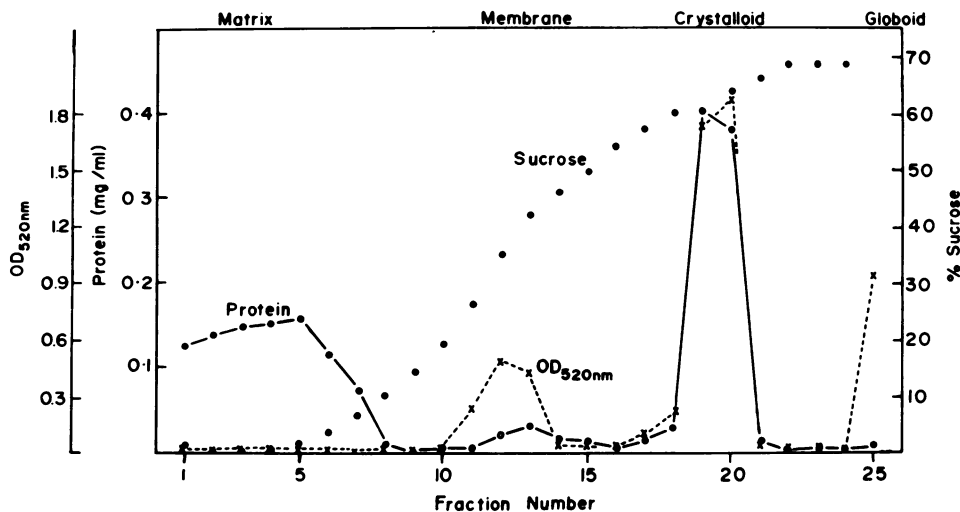


FIG. 3. Separation of the various suborganelle inclusions of lysed protein bodies in a sucrose gradient. The pellet of the gradient was resuspended and presented as fraction 25.

before, and the protein bodies again floated to their equilibrium density at 1.36 g/cm^3 .

Subfractionation of the protein bodies was performed by sucrose gradient centrifugation. Twenty ml of the isolated protein bodies in hexane- CCl_4 were mixed with 2 ml of H_2O , and all of the organic solvents were allowed to evaporate under N_2 . By this method, the protein bodies were broken. The preparation was applied to a sucrose gradient consisting of, from the bottom, 1 ml 68% (w/w) sucrose, a 9-ml gradient from 68% to 30% sucrose, 2 ml 15% sucrose, and 2 ml 5% sucrose. The gradient was centrifuged in a SW 27.1 rotor at 24,000 rpm for 6 hr, and fractionated.

Globoids of the protein bodies were also purified as described (20) with modification. Six g of deshelled dry castor beans were ground in 25 ml of 70% glycerol for 5 min at low speed in a VirTis 45 homogenizer. The homogenate was first incubated for 1 hr at room temperature with occasional stirring and centrifuged at $2,500g$ for 20 min. The pellet was resuspended in 95% ethanol and recentrifuged. The pellet was again resuspended in 95% ethanol and filtered through a Nitex cloth of $35 \mu\text{m}^2$ pore size. The filtrate was applied to a linear hexane- CCl_4 density gradient from 1.20 g/cm^3 to 1.58 g/cm^3 . The gradient was centrifuged at 10,000 rpm in a SW 27 rotor for 3 hr. The pellet was examined under the light microscope and found to contain only globoids with properties as described (20).

Lipid Determination. Each of the four suborganelle fractions—matrix, membrane, crystalloids, and globoids—obtained from the sucrose gradient, was diluted to 5% sucrose and centrifuged at $100,000g$ for 1 hr. The pellet was resuspended in 3 ml of H_2O and extracted overnight in 5 volumes of chloroform and methanol (2:1, v/v). The aqueous phase was separated and re-extracted with chloroform and methanol. The combined non-aqueous phase was washed once with H_2O and dried. The sample was resuspended in chloroform and analyzed by TLC. TLC plates were 0.25 mm thick prepared with Silica Gel G. Samples were spotted and run for 10 cm in a solvent of chloroform, methanol, and water (65:25:4, v/v) (11). Phospholipids were visualized by spraying the plates with 5% phosphomolybdate in ethanol and heating at 80 C for 10 min. Standards of phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine (Applied Science Laboratories Inc.) were run along with the samples for identification.

Gel Electrophoresis. Disc gel electrophoresis was performed in 7.5% polyacrylamide gels with a stacking gel and a discontinuous buffer system (25). Protein samples were incubated for 1 hr at room temperature in an equal volume of 0.01 M K-phosphate

buffer, pH 7.4, 1% SDS, and as appropriate, 1% β -mercaptoethanol. In the study of protein subunits, the protein bands were cut from the gel, extracted, treated with β -mercaptoethanol, and re-electrophoresed in SDS-acrylamide gels (25). After electrophoresis, the gels were stained for protein with Coomassie blue. Molecular weight standards (all from Sigma) were β -galactosidase (*Escherichia coli*), phosphorylase a (rabbit muscle), BSA, catalase, glyceraldehyde-3-P dehydrogenase, chymotrypsinogen, myoglobin, and Cyt c. Scanning of the gels was performed in a quartz cuvette in a Gilford gel scanner (model 2520) attached to a Gilford spectrophotometer and recorder.

Germination. Castor beans were soaked overnight in running tap water and germinated in moist vermiculite at 30 C in darkness. The endosperm at various ages was ground with a mortar and pestle in buffer (8) and the homogenate was filtered through a Nitex cloth of $35 \mu\text{m}^2$ pore size. The filtrate was layered onto a sucrose gradient composed of 28 ml of a 30 to 68% linear sucrose gradient, and centrifuged at 21,000 rpm for 2 hr with a SW 27 rotor. The gradient was fractionated and each fraction was assayed for *A* at 280 nm in the presence of 0.7 M NaCl, and for sucrose concentration with a Bausch and Lomb refractometer. Total protein was measured according to Lowry *et al.* (10) with BSA as the standard.

Purification of Lectins. The purification of ricin and phytohemagglutinin was performed according to Nicolson (14) using Sepharose 4B affinity chromatography and eluting the proteins with galactose. Purified ricin and phytohemagglutinin were also obtained from Miles Laboratories (Elkhart, Ind.).

RESULTS

Isolation of Protein Bodies. In the endosperm cells of dry castor bean, most of the cell volume is packed with protein bodies and lipid bodies (Fig. 1). When the total extract of dry castor bean obtained by grinding in hexane was centrifuged in a linear density gradient composed of hexane and carbon tetrachloride, a major protein peak was observed at density 1.36 g/cm^3 . This fraction was examined under the light microscope and found to contain intact protein bodies. There was a slight contamination by broken cell walls, the majority of which sedimented to a density of 1.41 g/cm^3 . Approximately 90% of the total protein was recovered at the fraction of protein bodies and the rest was present at the top of the gradient. The protein components at the top of the gradient resembled those of the isolated protein bodies in gel electrophoresis (data not presented) and showed no enrichment of any specific protein components. The proteins at the top of the gradient were probably

derived from broken protein bodies and there were little cytosol proteins present. After a flotation gradient centrifugation, the protein bodies were recovered again at density 1.36 g/cm^3 , which was free of contaminating cell wall. This fraction was composed of homogeneous intact protein bodies as observed under the light microscope (Fig. 2), and the isolated organelles with their inclusions resembled those *in situ* (Fig. 1).

Subfractionation of Protein Bodies. As observed *in situ* (Fig. 1), the protein body contained, in addition to a membrane and a protein matrix, inclusions of a protein crystalloid and several phytin globoids. These suborganelle compartments were separated from one another by centrifugation of the lysed protein bodies in a sucrose gradient. The gradient profile is shown in Figure 3. Two major bands at densities 1.15 g/cm^3 and 1.30 g/cm^3 were visible, as indicated by the diffraction of light at 520 nm. There was a pellet at the bottom of the centrifuge tube and it was resuspended and presented as fraction 25 in Figure 3. The four fractions—gradient supernatant, the fraction at density 1.15 g/cm^3 , the fraction at density 1.30 g/cm^3 , and the pellet, were found to represent four different compartments of the protein bodies.

The matrix proteins were soluble in water and remained at the top of the sucrose gradient. This fraction represented about half of the total protein of the protein bodies. Such an estimation is consistent with our observation on the relative amount of proteins distributed between the matrix and the crystalloids in numerous protein bodies viewed *in situ* (not shown). The solubilization of the matrix proteins, in contrast to the crystalloids, was also evidenced by the distribution of distinct protein components between the two fractions (next section).

The fraction at density 1.15 g/cm^3 had a high light diffraction at wavelength 520 nm, but a low protein content. When this fraction was diluted to 5% sucrose and centrifuged at $100,000g$ for 1 hr, a pellet was obtained. The resuspended pellet was extracted with chloroform and methanol and the components that were soluble in the organic solvents were analyzed by TLC. Three major spots of charged lipids were visualized and tentatively identified as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. When the other three suborganelle fractions were analyzed for phospholipids in an identical way, no phospholipid was detected in the gradient supernatant; there was a lesser amount of similar phospholipids in the fraction at density 1.30 g/cm^3 and a trace amount in the pellet. The small amount of membrane in the latter two fractions was probably due to contamination by intact protein bodies or protein body membrane dragged along by other particles during centrifugation. We thus identified the fraction at density 1.15 g/cm^3 as the membrane of the protein bodies.

Under the light microscope, the fraction at density 1.30 g/cm^3 was identified as the crystalloids of the protein bodies (Fig. 4). This fraction accounted for about half of the protein of the protein bodies (Fig. 3). The crystalloids retained their size and polyhedral shape as those observed *in situ*. The crystalloids were insoluble in H_2O but soluble in salt solution (0.37 M NaCl). When the salt in the solution was removed, the solubilized protein of the crystalloid reprecipitated out. Thus, the crystalloid proteins should be categorized as globulins.

The pellet at the bottom of the sucrose gradient represented particles that could migrate to a density greater than 1.46 g/cm^3 . Under the light microscope, this fraction was found to contain small spherical bodies of the same size as the globoids *in situ* (Fig. 5). These particles were not soluble in H_2O or dilute salt solution (0.37 M NaCl), but were soluble in dilute acid (2.5% trichloroacetic acid). The solubility of these particles is similar to that described previously on the castor bean globoids (20). We identified this fraction as the phytin globoids by their size, shape, high density, and solubility. The globoids were also isolated according to the method described (20), and found to have

similar properties and protein content as those that we isolated in sucrose gradient.

Protein Components of the Matrix and the Crystalloids of the Protein Bodies. The protein components of the total endosperm extract, the intact protein bodies, and their suborganelle fractions were analyzed by SDS-acrylamide gel electrophoresis (Fig. 6). The proteins in the total extract were resolved into protein components of diverse mol wt, and the gel pattern resembled that of the intact protein bodies. The resemblance was expected since the majority of the cellular proteins is confined to the protein bodies. For convenience of description, the protein bands are labeled as shown in Figure 6. In the intact protein bodies, bands 2, 3, and 6 were distinct, whereas bands 1, 4, and 5 were barely visible. The latter bands were resolved in the scanning profile (Fig. 7). By applying a higher concentration of the samples to the gel, band 1 was clearly resolved into three sub-bands, 1a, 1b, and 1c, but the separation of bands 2, 3, and 4 became indistinct due to overloading of proteins at that region. In the matrix fraction, bands 1, 2, 4, and 6 were visible and enriched, whereas bands 3 and 5 were completely missing. In direct contrast, in the crystalloid fraction, bands 3 and 6 were present, but all of the other bands were missing. Whereas the matrix proteins had very diverse mol wt, the crystalloid proteins were mainly composed of several proteins with mol wt between 50 and 60,000 daltons. There is no overlapping of the protein components between the matrix and the crystalloids. Thus, there is a clear compartmentation of the protein components within the protein bodies. The membrane fraction and the phytin globoid fraction contained little protein (Fig. 3), and therefore, substantial amounts of the two fractions had to be used for SDS-acrylamide gel electrophoresis. The membrane fraction showed a gel pattern similar to that of the total protein bodies, presumably due to the trapping of the matrix and the crystalloids by the

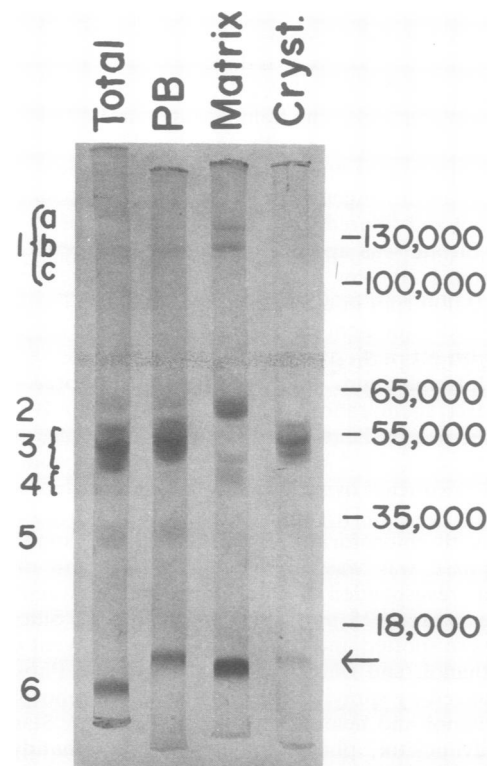


FIG. 6. Photograph of SDS-acrylamide gels after electrophoresis of the total castor bean extract, the isolated protein bodies (PB), the matrix, and the protein crystalloids (Cryst.). Numbers on the right hand side are estimated mol wt in daltons. Arrow indicates the position of the tracking dye which accounted for a small background in band 6.

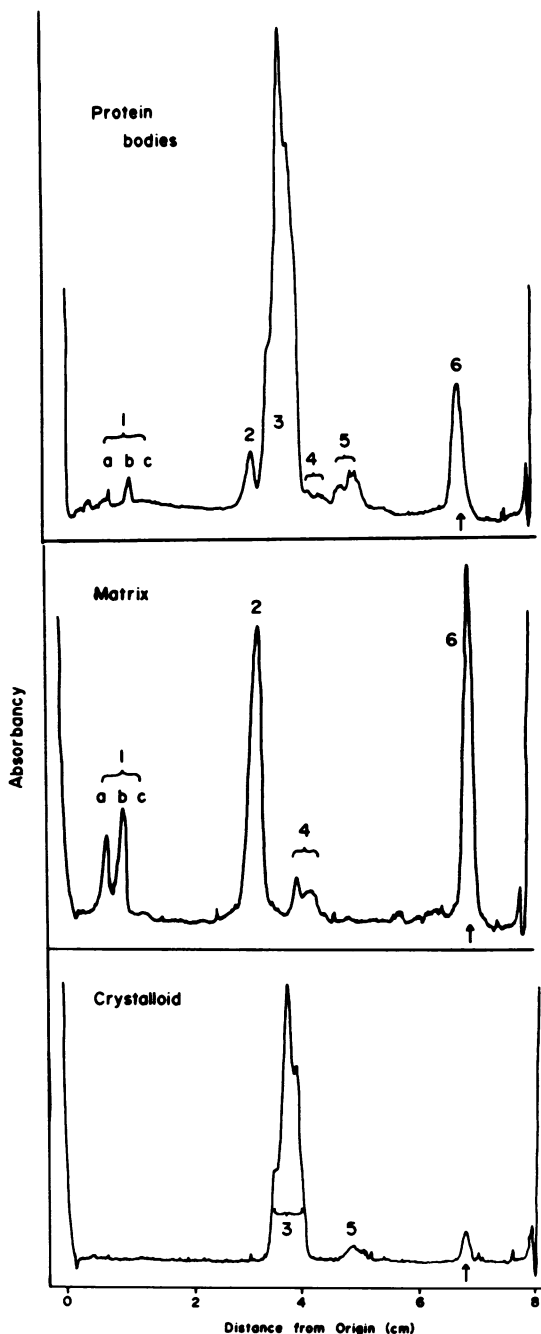


FIG. 7. Scanning profiles of proteins in SDS-acrylamide gels after electrophoresis of the total protein bodies, the protein crystalloids, and the matrix. Scanning was performed at 540 nm. Arrows indicate the position of the tracking dye which accounted for a small background in band 6.

broken membrane envelopes. The gel pattern of the phytin globoids resembled that of the crystalloids and was attributed to contaminating crystalloids.

Localization of Ricin and Phytohemagglutinin in the Matrix of the Protein Bodies. As shown in Figure 6, band 1 (sub-bands a, b, and c) and band 2 with mol wt around 120,000 daltons and 65,000 daltons, respectively, were restricted only to the matrix of the protein bodies. Two well known castor bean lectins, phytohemagglutinin and ricin, have been studied intensively (9, 13-15) and they have mol wt similar to those of band 1 and band 2, respectively. To see whether or not band 1 and band 2 are the phytohemagglutinin and ricin, the castor bean lectins were puri-

fied by the established method (14), and separated in the present system of gel electrophoresis (Fig. 8). The castor bean lectins were resolved into four bands, corresponding to bands 1 (sub-bands a, b, and c) and 2. These corresponding bands co-migrated together in electrophoresis (Fig. 8). Band 2 of the matrix was further confirmed to be ricin by its identity with commercial purified ricin in gel electrophoresis and by its subunit composition (14, 15) upon reduction with β -mercaptoethanol (data not shown). Band 1 (sub-bands a, b, and c) occurred at the region of the gel with mol wt around 130,000 daltons where mol wt estimation was not accurate. Its identity as the phytohemagglutinin was confirmed by its resemblance to the three sub-bands of commercial purified phytohemagglutinin in gel electrophoresis and by its subunit composition (14, 15) upon reduction with β -mercaptoethanol (data not shown). As reported in the specification from Miles Laboratories, the purified phytohemagglutinin had a mol wt of 120,000 daltons and gave only one protein band in a different system of gel electrophoresis. Thus, sub-bands 1a, 1b, and 1c may represent different forms of the phytohemagglutinin. We conclude that band 2 represents ricin and band 1 is the phytohemagglutinin, and they are both present within the matrix of the protein bodies.

Changes in Matrix Protein and Crystalloid Protein during Germination. The changes in individual protein components of the protein bodies during germination were studied by subjecting equal amount of extracts of endosperm at various ages to SDS-acrylamide gel electrophoresis. As shown in Figure 9, the intensity of the three major bands (bands 1, 2, and 6) of the matrix protein did not go through much change during the first 5 days of germination. In sharp contrast, band 3 of the crystalloids rapidly disappeared during the early stages of germination, with

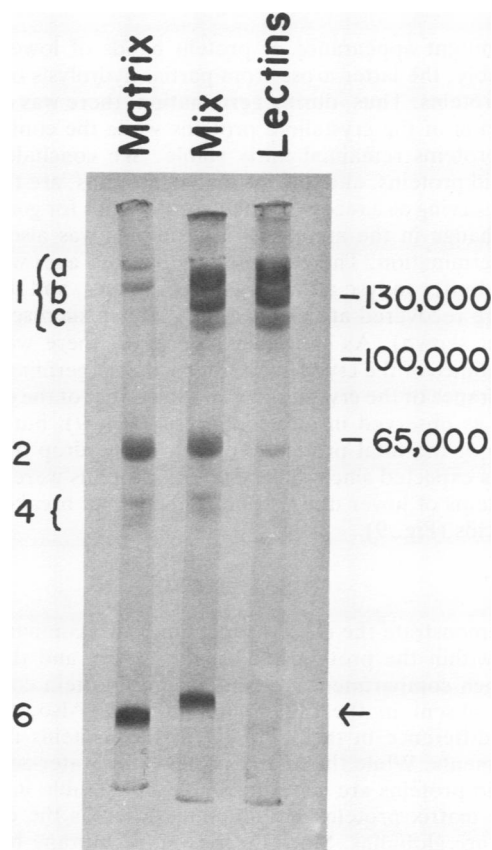


FIG. 8. Photograph of SDS-acrylamide gels after electrophoresis of the matrix of the protein bodies, the isolated castor bean lectins, and a mixture of the above two samples. Numbers on the right hand side are estimated mol wt in daltons.

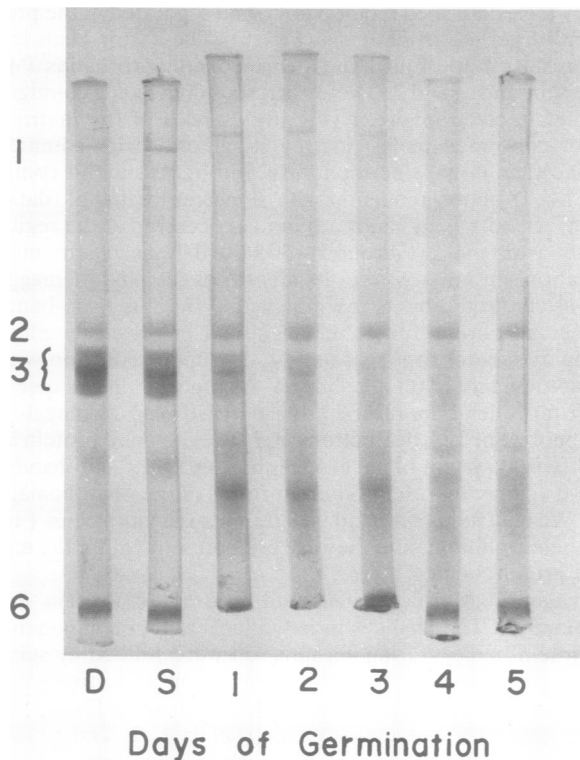


Fig. 9. Photograph of SDS-acrylamide gels after electrophoresis of the total extracts of castor bean endosperm at various days of germination. D and S denote dry and soaked beans, respectively.

a concomitant appearance of protein bands of lower mol wt. Presumably, the latter arose from partial hydrolysis of the crystalloid proteins. Thus, during germination, there was a selective degradation of the crystalloid proteins while the content of the matrix proteins remained fairly stable. We conclude that the crystalloid proteins, and not the matrix proteins, are the storage protein, serving as a reserve pool of amino acids for germination.

The change in the amount of crystalloids was also followed during germination. The endosperm at various ages was ground in dilute buffer to lyse all of the protein bodies, and the crystalloids were recovered at density 1.30 g/cm^3 in sucrose gradients (data not shown). As shown in Figure 10, there was a rapid disappearance of the crystalloids after 1 day of germination. The disappearance of the crystalloids paralleled that of the crystalloid proteins as observed in electrophoresis (Fig. 9), but preceded the drop of the total protein. This lag in the drop of the total protein is expected since the crystalloid proteins were degraded into proteins of lower mol wt before their final breakdown into amino acids (Fig. 9).

DISCUSSION

We demonstrate the clear distinction of two compartments of protein within the protein bodies: the matrix and the crystalloids. Each compartment contains distinct protein components that are absent in the other compartment. Also, there is a marked difference in the solubility of the proteins in the two compartments. While the matrix proteins are water-soluble, the crystalloid proteins are water-insoluble but soluble in salt solution; the matrix proteins are albumins whereas the crystalloid proteins are globulins. Since there is no membrane barrier between the two compartments, it is noteworthy to see how the various protein components are compartmentalized during seed formation. Perhaps the protein components are separated into the two compartments solely by their physical properties (e.g.

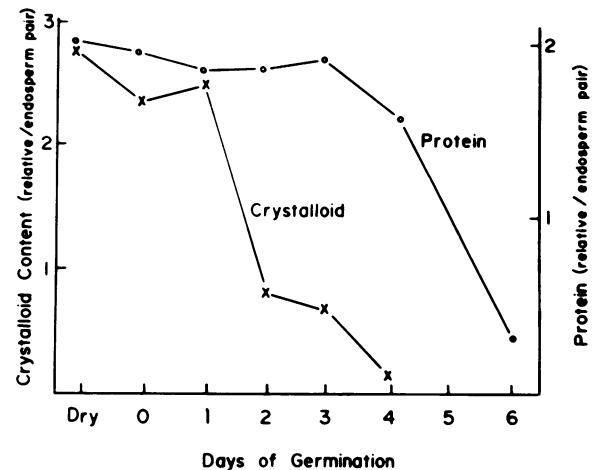


Fig. 10. Developmental changes in the total protein and the amount of isolated protein crystalloids in the endosperm of castor bean during germination. 100% total protein represents 200 mg/endosperm pair.

solubilities, charges, and ability to form crystals). Alternatively, they may be compartmentalized by a temporal difference in their synthesis during seed formation. The interrelationship between the two compartments is also interesting in view of their difference in physiological roles. During germination, the crystalloid proteins are degraded into smaller molecules, presumably by the action of endopeptidases, before complete hydrolysis occurs. Thus, they function as the storage proteins. On the other hand, the matrix proteins do not serve as the storage proteins, and the content remains fairly stable during the active period of protein hydrolysis. Since it is well documented (7, 16) that many hydrolytic activities are associated with the protein bodies, the matrix proteins may well be the hydrolytic enzymes responsible for the degradation of storage protein and phytin.

Lectins are proteins with widespread occurrence in seeds, but their physiological roles are unknown. It was suggested that in legume cotyledons, the lectins are localized mainly in the cytosol (4). The localization of the castor bean lectins within the matrix of the protein bodies (present work) and their known properties (9, 13-15) may imply that they have metabolic functions. Ricin has been shown to be a strong protein synthesis inhibitor, acting catalytically on the 60S subunits of eucaryotic ribosomes. The localization of this toxic protein in the protein bodies, which will become the cell vacuole, may explain its nontoxic effect on the *in vivo* protein synthesis of the endosperm cell.

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