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Accumulation and Subcellular Localization of α-Galactosidase-Hemagglutinin in Developing Soybean Cotyledons[1]

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ABSTRACT

We have investigated the accumulation and intracellular localization of soybean (*Glycine max* [L.] Merr. cv Forrest) α-galactosidase-hemagglutinin during seed development. Cotyledon tissue was embedded in Lowicryl K4M and immunocytochemical localization was accomplished through treating thin sections with α-galactosidase antisera followed by indirect labeling with protein A coupled to colloidal gold. Gold particles were localized on the Golgi apparatus and protein bodies. We interpret this to indicate that α-galactosidase-hemagglutinin is transferred to and transported through the Golgi apparatus and finally deposited within the protein body by a Golgi apparatus-mediated process.

α-Galactosidase (EC 3.2.1.22) is widely distributed among plant species and is generally considered to participate in the degradation of reserve raffinose-type oligosaccharides and cell wall galactomannans (for review, see 25). The enzyme, depending on its source, displays a preference for one of these substrates (for review, see 25). The α-galactosidase from legume seed cotyledons exhibits a substrate preference for the raffinose-type oligosaccharide and shows only minimal activity toward galactomannans (5, 10). Thus, the presumed function of the cotyledon α-galactosidase is to degrade the raffinose-type oligosaccharides during germination and early seedling growth (18).

α-Galactosidase from mature soybean seeds is a tetrameric protein (10) with a mol wt of 160,000 D at pH 4.0 (4). At pH 7.0, the protein dissociates into subunits with mol wt of 38,000 and 40,000 D (4). Although both the monomeric and tetrameric forms are enzymically active, they display different kinetic properties (4). At pH 4.0, the enzyme displays a transitory type of hemagglutinin activity and is referred to as α-galactosidase-hemagglutinin (4). Whether the hemagglutinin activity is physiologically relevent is unknown. The α-galactosidase from soybean is virtually identical in biochemical and physical properties to α-galactosidases from the cotyledons of other legume species which may or may not display hemagglutinin activity (8, 9). Soybean α-galactosidase-hemagglutinin is discrete from the well-characterized seed lectin, SBA[2] (4). Because of its lectin-like properties and because it is immunologically related to legume lectins which bind galactose (8, 9), the α-galactosidases of mung bean and soybean seeds have been of particular interest to lectin researchers.

The present study employs high-resolution electron microscope immunocytochemical observations to examine the developmental accumulation and intracellular localization of α-galactosidase in soybean cotyledons. Our findings indicate that soybean α-galactosidase is localized in protein bodies within storage parenchyma cells. We also demonstrate that α-galactosidase is localized in the Golgi apparatus of developing seed storage parenchyma cells supporting the proposal that the Golgi apparatus mediates the deposition of the legume protein body matrix (2, 3, 6, 12, 13, 21). The present observations are the first evidence that a specific protein body acid hydrolase is deposited in the protein body by a Golgi apparatus-mediated process.

MATERIALS AND METHODS

Plant Material. *Glycine max* Merr. cv Forrest seeds were surface-sterilized with 10% commercial bleach, imbibed overnight with distilled H_2O, and grown in potting soil in the glass house. Seed pods were harvested at various times after pod set and the seeds removed and weighed. Seed weight has previously been shown to be a valid method for assigning developmental stages of soybean seeds (19).

α-Galactosidase Assay. One cotyledon from seeds weighing 25 to 300 mg fresh weight in 25 mg ± 5.0 increments, was ground with a mortar and pestle in 1 ml grinding buffer (1.0 M NaCl, 0.1 M Tris-HCl [pH 8.0], 5 mg/ml Nonidet NP-40 (Particle Data Laboratories, Elmhurst, IL). The homogenate was centrifuged (10,000 rpm in an SS34 rotor for 10 min at 4°C; Sorvall RC 2B centrifuge) and the supernatant solution assayed for α-galactosidase activity by incubating 20 μl in 1 ml 2 mM *p*-nitrophenyl-α-galactopyranoside (Sigma) and 0.1 M sodium acetate [pH 5.0] for 90 min at 30°C. The reaction was terminated by the addition of 1 ml 1.0 M sodium bicarbonate [pH 9.0]. The *p*-nitrophenol released by α-galactosidase was measured by its *A* at 420 nm.

SDS-PAGE and Immunoblots. Midmaturation soybean cotyledons (150 mg) were prepared for SDS-gel electrophoresis as described by Meincke *et al.* (19). SDS-PAGE was accomplished with a 12% resolving gel and 5% stacking gel using the discontinuous buffer system of Laemmli (16). Electroblots were obtained by the method of Towbin *et al.* (27). Electroblots were stained either with Coomassie blue to visualize total proteins or processed for immunostaining by the minor modification of the method of Knecht and Dimond (15). The modification of their method included the use of 50% fetal calf serum for blocking and the substitution of Tris-buffered saline for PBS. The primary antiserum was used at a dilution of 1:500.

Immunocytochemistry. Cotyledons from seeds weighing 150 mg fresh weight were sliced into 1 mm^3 cubes and fixed in 4% (w/v) depolymerized paraformaldehyde, 2% (v/v) glutaraldehyde (Polysciences, Inc., Warrington, PA), 0.1 M K-phosphate buffer (pH 7.4) for 48 h at 7°C. The tissue was rinsed in 0.1 M K-phosphate buffer (pH 7.4) and dehydrated in DMF (Aldrich Chemical Co., Milwaukee, WI) using 25%, 50%, 75%, and 90%

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[2] Abbreviations: SBA, soybean agglutinin; DMF, *N*, *N*-dimethylformamide.

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(v/v) steps for 1 h each at 20°C. Tissue pigments were extracted in the 75% and 90% dehydration steps. A final 90% wash for 12 h greatly facilitated resin infiltration. The dehydrated tissue was infiltrated with Lowicryl K4M resin (Polysciences, Inc., Warrington, PA), containing 85% (w/w) monomer, 15% (w/w) crosslinker, and 0.5% (w/w) benzoin methyl ether. Infiltration was carried out at 20°C for 12 h in a 1:1 mixture of resin and 90% (v/v) DMF and then for 2 d in 100% resin. Aluminum foil was wrapped around the sample containers to minimize exposure to light.

Tissue samples were loaded into BEEM capsules (Ted Pella Co., Tustin, CA), filled with resin, and capped to preclude contact with air. The blocks were polymerized by illumination with longwave UV light (General Electric F15T8-BLB) for 12 h at −10°C, followed by 48 h at 20°C. The blocks were stored in sealed containers to avoid hygroscopic softening.

Tissue sections, approximately 0.1 μm thick, were mounted on uncoated 300 mesh nickel grids. Immunocytochemical labeling was accomplished by immersing the grids in whole rabbit antisera specific for α-galactosidase, diluted 1:10 with TBST buffer (0.5 M NaCl, 20 mM Tris-HCl [pH 7.4], 1% w/v Tween-20) for 15 min at 20°C. As a control, other grids were incubated in preimmune whole rabbit serum diluted 1:10 with TBST. The whole rabbit antiserum was raised against mung bean α-galactosidase (16) and is pooled high titer sera. The grids were washed with TBST buffer, and then labeled in a 10 μl drop of protein A coupled to 20 nm colloidal gold prepared by the method of Roth *et al.* (26). The grids were labeled for 5 min at 20°C, washed with TBST buffer, and then with distilled H_2O. Following a final contrasting stain with 5% (w/v) aqueous uranyl acetate (20 min at 20°C), the grids were examined and photographed using a Philips 400 electron microscope.

RESULTS

Accumulation of α-Galactosidase during Seed Development. Soybean seeds were collected at various stages of development and analyzed for α-galactosidase (Fig. 1). The enzyme is present at 25 mg fresh weight and accumulates linearly with seed growth. The midpoint of α-galactosidase accumulation coincides with the midpoint of seed growth (150 mg fresh weight). The specific activity of α-galactosidase remained constant during seed development at 10 nmol *p*-nitrophenyl released/min·mg fresh weight.

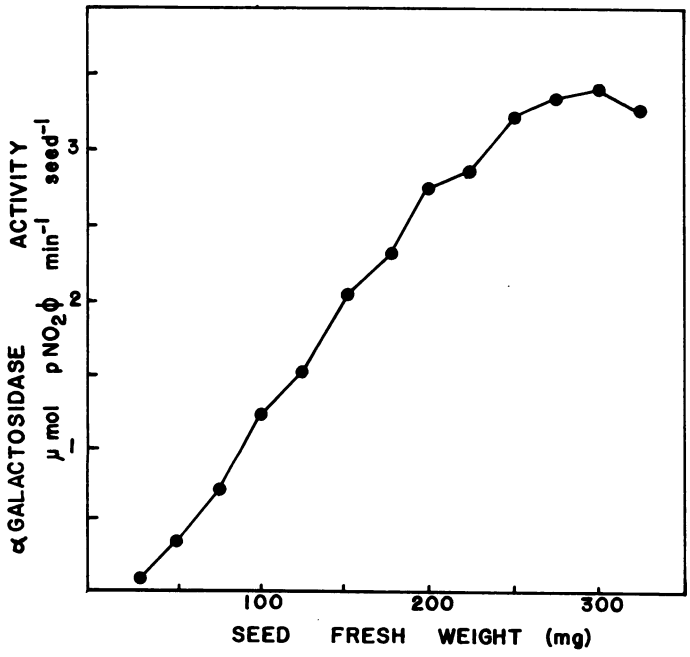


FIG. 1. Developmental accumulation of total seed α-galactosidase activity.

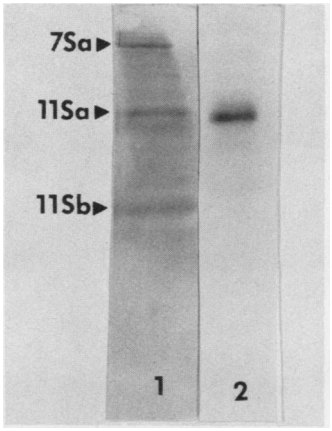
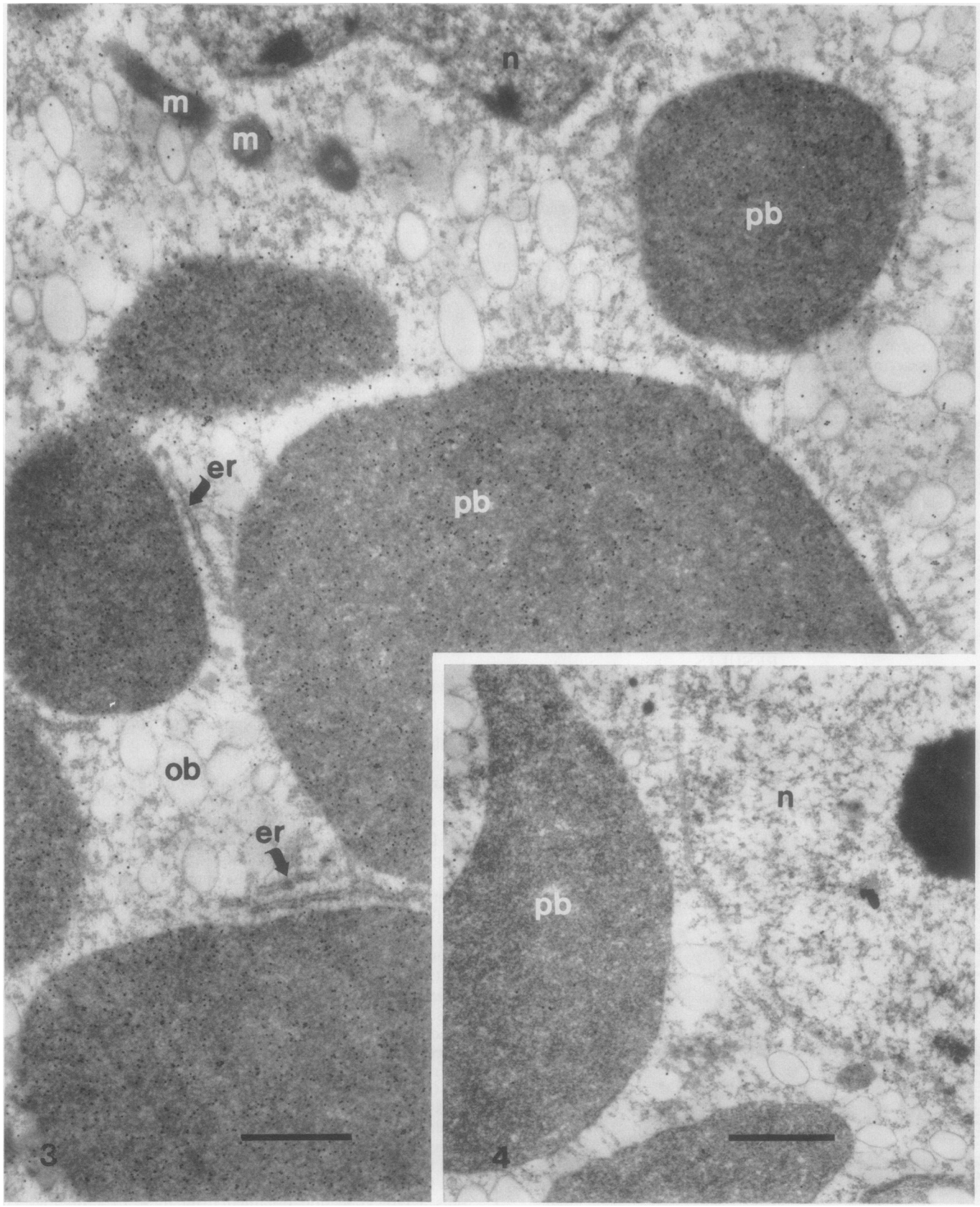


FIG. 2. Parallel electroblots of the total proteins of mid-maturation soybean cotyledons. Lane 1, Coomassie blue-stained electroblot showing the distribution of the major 7sa and 11s acidic (11sa) and basic (11sb) storage protein subunits. Lane 2, Antibody-stained electroblot showing that a single protein band of 36,000 M_r is stained with α-galactosidase antisera.

Antisera Specificity. The α-galactosidase antisera used here were raised against purified mung bean enzyme (7). The antisera cross-react with purified soybean α-galactosidase in double-diffusion assays resulting in a line of identity (4). The specificity was further examined using the immunoblot technique. A crude midmaturation cotyledon extract was subjected to SDS-PAGE and electroblotted onto nitrocellulose paper. Parallel blots were stained with either Coomassie blue or probed with α-galactosidase antisera and stained by the method of Knecht and Dimond (15) (Fig. 2). The resulting staining reaction visualized a single band of 36,000 M_r. The M_r obtained is similar to that of purified α-galactosidase (4). No other major or minor protein was stained, demonstrating high specificity of the antisera preparation.

Structural Preservation. Soybean cotyledon tissue was successfully embedded in Lowicryl K4M by the procedures described here and elsewhere (12). Cotyledon tissue embedded in Lowicryl K4M presents a different structural perspective than is obtained using more conventional electron microscope techniques. We have discussed this in detail elsewhere (12), but reiterate some general observations here. The absence of osmium tetroxide postfixation, followed by the preparative use of organic solvents, such as DMF, resulted in the extraction of oil and pigments (unpublished observations). Subcellular structures such as the ER (Figs. 3 and 5), Golgi apparatus (Figs. 5 and 6), protein bodies (Figs. 4, 5, and 7), oil bodies (Figs. 3–7), and nucleus (Figs. 3 and 4) were easily identified in Lowicryl sections. No obvious swelling or rupture of subcellular organelles was observed. The organelles did not appear as electron dense or contrasty as the tissue prepared using conventional procedures.

α-Galactosidase Localization in the Protein Bodies. Thin sections from 150 mg fresh weight soybean seed, the midpoint of α-galactosidase accumulation, were labeled with rabbit antibodies raised against α-galactosidase followed by protein A coupled to 20 nm colloidal gold. These treatments revealed specific gold labeling of several intracellular components. Figure 3 presents a typical section obtained from these experiments. The protein bodies were densely labeled. The distribution of gold particles over the protein bodies was uniform within an individual protein body, and among protein bodies within a cell. Differences observed between cells correlated directly with the relative electron density of protein deposits, the more dense protein



FIGS. 3 and 4. Figure 3, Portion of a storage parenchyma cell section labeled with anti α-galactosidase whole rabbit sera followed by protein A-colloidal gold. The protein bodies (pb) are specifically labeled. Also shown is a portion of the nucleus (n), mitochondria (m), endoplasmic reticulum (er), and oil bodies (ob) which are not specifically labeled. Bar = 1 μm × 20,000. Figure 4, Control section labeled with whole rabbit preimmune sera followed by protein A-colloidal gold. Note that there is a sparse level of gold label over the protein body (pb). Bar = 1 μm × 20,000.

deposits displayed a higher density of gold label. Other intracellular organelles, *i.e.* nucleus, mitochondria, and oil bodies had a sparse level of gold particle labeling identical to that observed in control.

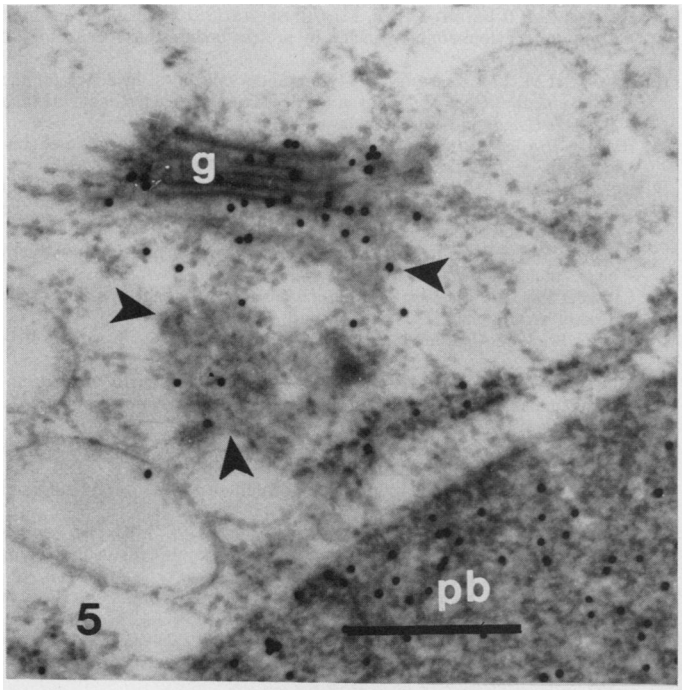
Comparison of α-galactosidase antibody-treated sections with control sections, in which normal rabbit serum was substituted for specific antiserum, demonstrated the high specificity of the reaction. Control sections had a sparse level of gold label on the protein body as well as other subcellular organelles (Fig. 4).

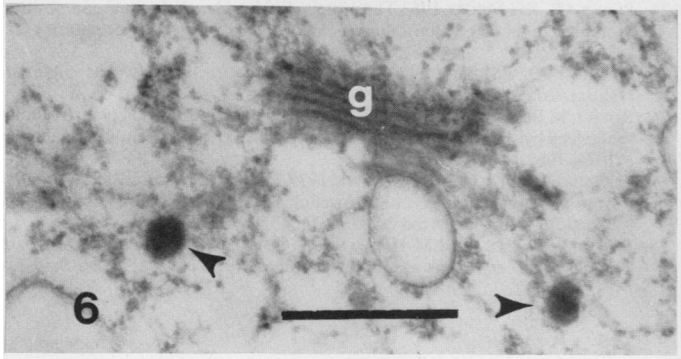
α-Galactosidase Localization in the Golgi Apparatus. Labeling thin sections or midmaturation storage parenchyma cells with anti-α-galactosidase rabbit sera revealed that α-galactosidase was detected in the Golgi apparatus. The entire Golgi apparatus cisternal stack was specifically labeled with colloidal gold particles (Fig. 5). No difference in the density of gold label across the Golgi apparatus cisternal stack was apparent. Comparison of sections incubated with the specific antisera (Fig. 5) and with preimmune serum (Fig. 6) confirmed the specificity of the immunocytochemical label of the Golgi apparatus.

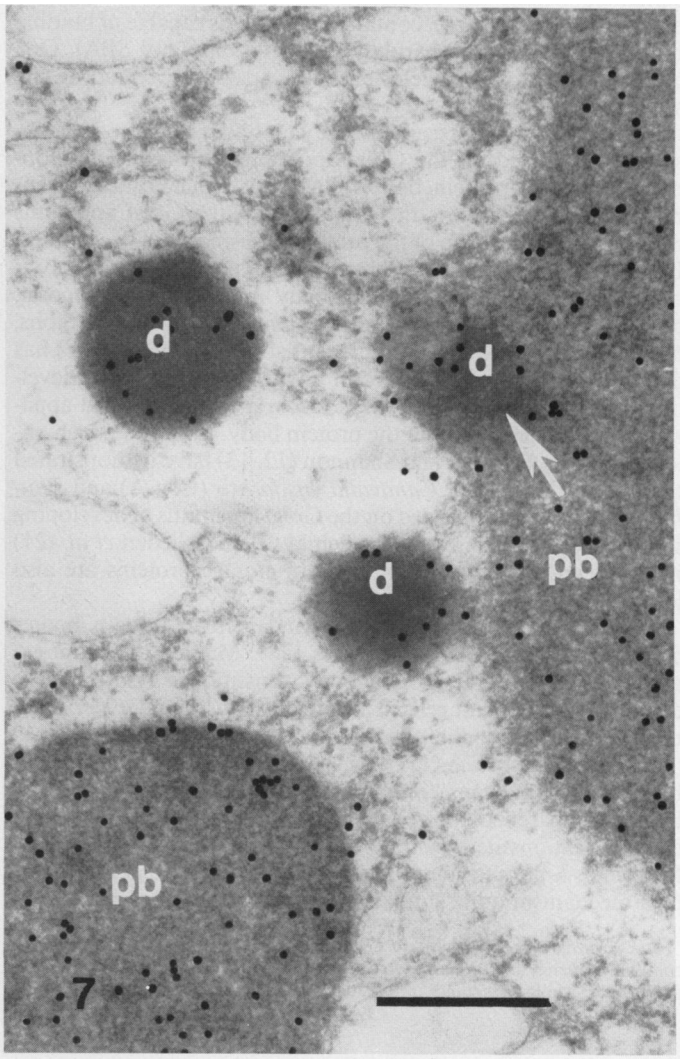
Immunolabeling of Dense Bodies. Electron dense bodies (0.5–1.0 μm in cross-section) were also observed (Fig. 7). The structures were intermediate in cross-section size between the Golgi apparatus secretion vesicles (0.1 μm; Fig. 6) and protein bodies (>5.0 μm). The electron density of these structures more closely resembled that of the Golgi apparatus secretion vesicle (Fig. 6, arrows) than that of the protein body (Fig. 7). The dense bodies were labeled for the presence of the α-galactosidase antigen (Fig. 7). The concentration of gold particles on the dense bodies was similar to that on adjacent protein bodies. The ontogeny of these structures remains unknown. The dense bodies were sometimes observed to be continuous with the protein body (Fig. 7, arrow). Whether the dense body shown was in the process of fusing to, or emerging from, the protein body remains to be determined.

DISCUSSION

The immunocytochemical observations presented here indicate that the intracellular site of α-galactosidase accumulation is







FIGS. 5–7. Figure 5, Golgi apparatus (G) in a storage parenchyma cell labeled with anti α-galactosidase rabbit sera followed by protein A-colloidal gold. The entire Golgi apparatus cisternal stack and adjacent protein body (pb) are labeled with gold particles. Additional label is apparent on electron dense vesicles located between the Golgi apparatus and protein body (arrowheads). Bar = 0.5 μm × 57,000. Figure 6, Control section incubated with rabbit preimmune serum followed by protein A-colloidal gold. This micrograph shows a Golgi apparatus (g) of comparable morphology to that in Figure 4. Note the absence of gold label. Unlabeled electron dense vesicles are also shown (arrowheads). Bar = 0.5 μm × 57,000. Figure 7, Electron dense bodies (d) in the cytoplasm near the protein bodies (pb). Dense bodies are also observed to be continuous with the protein body (arrow). The dense bodies are labeled for the presence of α-galactosidase; the concentration of gold particles over the dense bodies is similar to that over the adjacent protein bodies. Bar = 0.5 μm × 57,000.

the protein storage vacuole (protein body) within the storage parenchyma cell. A previous biochemical study detected α-galactosidase in lupine protein bodies isolated by a nonaqueous procedure (23). Previous electron microscope immunocytochemical studies of developing legume seeds have examined the intracellular distribution of storage proteins (1, 3, 21) and lectins (12, 13). These proteins constitute a major fraction of the total seed protein. Soybean-galactosidase, however, is a minor protein which constitutes only about 0.05% of the total protein based on specific activities (4). The present study demonstrates the sensitivity of electron microscope immunocytochemistry of a seed protein antigen far less abundant than storage proteins and lectins.

The *in vivo* substrate of soybean α-galactosidase is the raffinose-type oligosaccharide (18). A major portion of the total raffinose-type oligosaccharide in cotton and lupine seeds has been shown to be present in isolated protein bodies (20, 24). Raffinose and stachyose are also competitive inhibitors of SBA (22). Horisberger and Vonlanthen (14) have shown that SBA is contained within the cotyledon cell protein bodies. Soybean protein bodies, therefore, contain two proteins capable of binding raffinose-type oligosaccharides (α-galactosidase and SBA). One of these proteins (α-galactosidase) is capable of degrading the oligosaccharide.

The observations presented here demonstrate that α-galactosidase is localized on the Golgi apparatus during the developmental stage of protein body filling and α-galactosidase accumulation. This supports the proposal that the Golgi apparatus mediates the deposition of the protein body matrix. Dieckert and Dieckert (6) first proposed that the Golgi apparatus is involved in the deposition of the protein body matrix. Their proposal, based on an interpretation of conventional electron micrographs, has been confirmed by several recent studies. Chrispeels (2) has demonstrated that the seed lectin phytohemagglutinin of developing *Phaseolus vulgaris* seeds is fucosylated in the Golgi apparatus prior to deposition in the protein body. Immunocytochemical studies by Herman and Shannon (12, 13) have demonstrated that the seed lectins of *Canavalia ensiformis* (Con A) and *Bauhinia purpurea* are localized on the Golgi apparatus of developing seeds. Similarly, Craig and Goodchild (3) and Nieden *et al.* (21) have shown that vicilin and legumin storage proteins are also localized on the Golgi apparatus.

In addition to storage proteins and lectins, protein bodies contain numerous acid hydrolases (28). Matile (17) proposed that the vacuole and protein body constitute a lysosome-like compartment in plant cells. This proposal has found experimental confirmation in the observations of Van der Wilden *et al.* (28) and Herman *et al.* (11) which demonstrated that the protein bodies of mung beans contain acid hydrolases and function as the storage parenchyma cell's autophagic compartent. The present observations demonstrate that a specific protein body acid hydrolase, α-galactosidase, is deposited by the Golgi apparatus by a mechanism quite similar to that of animal lysosomes.

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