Protein bodies of mung bean cotyledons as autophagic organelles

(acid hydrolase/autophagy/lysosome)

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ABSTRACT We present evidence that protein bodies constitute the principal lytic compartment in storage parenchyma cells of mung bean cotyledons and propose that they play a role in cellular autophagy. We developed a method to isolate protein bodies by incubating tissue slices with cell wall-degrading enzymes and fractionating the cellular organelles on a Ficoll gradient. About 75–80% of the protein bodies present in the protoplasts were recovered intact in a band at the 5/25% Ficoll interface. This band contained a similar proportion of the cellular α -mannosidase, *N*-acetyl- β -glucosaminidase, ribonuclease, acid phosphatase, phosphodiesterase, and phospholipase D. β -Amylase was present in the cells but not in the protein bodies. Ultrastructural observations showed that on the 3rd day of seedling growth protein bodies contain small vesicles (0.3–1.0 μ m) with a cytoplasmic content (ribosomes, membrane vesicles, mitochondria). Later in seedling growth these vesicles appeared empty. We believe that these are autophagic vesicles resulting from invaginations of the protein body membrane and that their cytoplasmic contents are digested by the acid hydrolases present in the protein bodies.

The protein reserves of many seeds are stored in special tissues and contained in protein bodies, large (2–10 μ m in diameter) spherical organelles bounded by a single membrane. Although most of the content of the protein bodies can be accounted for by the reserve proteins, these organelles also contain phytin, lectins, and certain acid hydrolases (for a recent review see ref. 1). In cotyledons of leguminous seeds, formation of protein bodies occurs during seed ripening. Reserve proteins are synthesized on rough endoplasmic reticulum (2) and accumulate first in the central vacuole and later in protein bodies formed *de novo* (for a review see ref. 3). During seedling growth the reserve proteins are catabolized within the protein bodies. When the protein bodies appear “empty,” their limiting membranes fuse to form a central vacuole (4). These observations indicate a clear ontogenetic relationship between protein bodies and vacuoles in legume cotyledons and suggest that protein bodies may be considered as storage-protein-filled vacuoles.

Matile (5) proposed more than 10 years ago that protein bodies have three functions: (i) they contain reserves, especially protein and phytin; (ii) upon germination they become hydrolytic compartments in which the hydrolysis of the reserves occurs; (iii) the protein bodies are the cellular compartments in which nonstorage macromolecules are broken down by acid hydrolases. The first function has been amply documented for many plant species either by analyzing the contents of isolated protein bodies or with cytochemical methods (1). The second function was deduced from ultrastructural observations showing that the electron-dense protein matrix disappeared

during seedling growth, while the membrane surrounding the protein body remained intact (4). Recently, biochemical evidence has been obtained which confirms that the hydrolysis of reserve protein occurs within the protein bodies (6–8). No evidence has yet been presented for the degradation within the protein bodies of macromolecules other than the stored reserves, and this function, postulated by Matile (5), is as yet undocumented (1).

The central vacuole of the plant cells contains many acid hydrolases (9–12), and there is now considerable evidence that it plays a role in autophagy and the breakdown of cellular organelles and macromolecules (for a review see ref. 13). Nishimura and Beevers (11) have shown that vacuoles derived from the fusion of “empty” protein bodies also contain numerous hydrolases. There is, however, little information about the enzyme content of the protein-filled protein bodies of ripening or germinating seeds. Presented here is evidence that such protein bodies contain many acid hydrolases and constitute the principal lytic compartment in the storage parenchyma cells. We suggest on the basis of morphological observation that protein bodies play a role in cellular autophagy during seed germination.

MATERIALS AND METHODS

Seeds of mung bean [*Vigna radiata* (Linnaeus) Wilczek] obtained from a local dealer were sterilized in 10% commercial bleach and germinated in the dark in moist vermiculite as described (14).

Isolation of Protoplasts and Protein Bodies. Forty cotyledons were cut in 1-mm slices and incubated in a solution of 1% Macerase (Calbiochem), 1% Cellulysin (Calbiochem), and 0.1% Polycillin in mannitol medium [0.6 M mannitol/10 mM sodium 2-(*N*-morpholino)ethanesulfonate buffer at pH 5.5]. After 10–12 hr of incubation at 25°C with gentle shaking the macerated tissue was washed six times with mannitol medium containing 0.1 mM EDTA to remove the digestive enzymes. The macerated tissue was resuspended in this medium to free protoplasts from the tissue slices and was carefully strained through nylon cloth (pore width approximately 500 μ m) to remove large tissue fragments. The protoplasts were allowed to settle and the supernatant was sucked off. The protoplast sediment was resuspended in mannitol medium containing 0.5 mM EDTA and pressed through a metal screen (pore width approximately 250 μ m). The material that passed through the screen contained some unbroken protoplasts and starch grains (both were allowed to settle out), and all the cytoplasmic organelles including protein bodies. The supernatant (after settling out) was loaded on a discontinuous Ficoll gradient (5% over 25% Ficoll in mannitol medium with 0.5 mM EDTA), which was centrifuged for 20 min at $9.0 \times g$ (200 rpm in a Sorvall RC-3 centrifuge with an HL-8 rotor). The gradient was fractionated in 0.5-ml fractions and each fraction was assayed for enzyme activity.

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Enzyme Assays. α -Mannosidase and *N*-acetyl- β -glucosaminidase were assayed at pH 5.0 with *p*-nitrophenyl derivatives as substrates (14). Carboxypeptidase was measured at pH 5.0 with *N*-carbobenzoxy-L-phenylalanine as substrate (14). Ribonuclease was determined at pH 5.0 by the method of Ambellan and Hollander (15). Phospholipase D was assayed at pH 5.0 as described in our earlier paper (16), with radioactive phosphatidylcholine as substrate. Acid phosphatase and phosphodiesterase were assayed at pH 5.0 with *p*-nitrophenyl derivatives as substrates. Proteinase (vicilin peptidohydrolase) was measured at pH 5.0 with Azocoll as substrate (14). β -Amylase was measured at pH 5.0 by determining the release of reducing sugars from soluble starch as described by Bernfeld (17). Leucine aminopeptidase activity was determined at pH 7.0 with leucine *p*-nitroanilide as substrate (14). NADH-cytochrome *c* reductase was assayed as described (2). Catalase was measured according to the procedure of Beers and Sizer (18). Protein was determined by the method of Lowry *et al.* (19), with bovine serum albumin as a standard.

Electron Microscopy. Small pieces (1 mm³) of cotyledon tissue were fixed for 12 hr at 7°C in 50 mM sodium cacodylate, pH 7.4, containing 4% (wt/wt), formaldehyde and 2% (wt/wt) glutaraldehyde. The pieces of tissue were rinsed in the fixation buffer, then postfixed in 1% osmium tetroxide for 18 hr at 7°C. The material was dehydrated in a graded acetone series, transferred to propylene oxide, and embedded in araldite. The specimens were sectioned with glass knives and the sections were stained in uranyl acetate (10 mg/ml in water) and alkaline lead citrate (5 mg/ml). The sections were visualized with a JEOL 100 S or a Phillips 300 electron microscope.

RESULTS AND DISCUSSION

Isolation of Protein Bodies. Fig. 1A shows protoplasts obtained after the overnight incubation of cotyledon slices with cell wall-degrading enzymes at room temperature. The enzymatic digestion did not cause the release of the fragile protoplasts into the incubation medium until the partially digested tissue slices were lightly shaken in the morning. The protoplasts were washed free of the cell wall-degrading enzymes by repeated (six times) washing with mannitol medium containing 0.1 mM EDTA. Each time the protoplasts were allowed to settle out and the supernatant was removed. Preparations of protoplasts always contained free starch grains (Fig. 1A) because the free starch grains and the starch-filled protoplasts settled out together when the protoplasts were washed free of the cellulytic enzymes. Although the commercial preparations of cell wall-degrading enzymes are rich in proteinase activity (12), no such proteinase activity was present in the isolated protoplasts (data not shown). This observation indicates that the protoplasts were not contaminated by hydrolytic enzymes originating in the preparations of cell wall-degrading enzymes.

Washed protoplasts were broken mechanically and layered (after settling out of the starch grains) on a discontinuous Ficoll gradient. Centrifugation resulted in the formation of a band at the 5/25% Ficoll interface. Examination of this fraction with the light microscope showed it to be a homogeneous preparation of large round organelles. When neutral red was added to this preparation these organelles accumulated the dye. Examination with the electron microscope showed the fraction to be primarily intact protein bodies (Fig. 1B). Contamination of this

FIG. 1. (A) Light micrograph of protoplasts isolated from slices of mung bean cotyledons digested with cell wall-digesting enzymes. Starch grains (St) are clearly visible in the cells. Free starch grains contaminate protoplast preparations because starch grains and protoplasts settle out together when preparations are washed to remove cell wall-degrading enzymes. (B) Electron micrograph of protein bodies (PB) isolated from protoplasts on Ficoll gradients. Sharp boundaries of the protein bodies indicate protein body membranes are intact. Preparations always contain membrane profiles (M) as contaminants. These are thought to be protein body membranes because biochemical analysis shows the preparation to be largely free of cytoplasmic contaminants.

Table 1. Distribution of enzyme activity between protein body fractions and supernatant ("load") fractions on a discontinuous Ficoll gradient

Enzyme	% in fraction	
	Supernatant	Protein bodies
α -Mannosidase	21.6	78.4
Carboxypeptidase	17.9	82.1
<i>N</i> -Acetyl- β -glucosaminidase	24.7	75.3
Acid phosphatase	21.3	78.7
Phosphodiesterase	22.9	77.1
Ribonuclease	24.5	75.5
Phospholipase D	21.5	78.5
Leucine aminopeptidase	95.3	4.7
β -Amylase	>99.9	<0.1
NADH-cytochrome <i>c</i> reductase	>99.9	<0.1

Protoplasts were prepared from cotyledons of seeds 36 hr after the start of imbibition and the ruptured protoplasts were layered on a 5/25% discontinuous Ficoll gradient that was centrifuged at $9.0 \times g$ for 20 min.

fraction by other organelles was determined with marker enzymes and by electron microscopy. We determined the distribution of leucine aminopeptidase (cytosol marker), NADH-cytochrome *c* reductase (endoplasmic reticulum marker), and catalase (microbody marker) and found more than 95% of the activity of each enzyme in the "load" portion of the gradient (Table 1). Examination of the fractions by electron microscopy indicated the absence of mitochondria but the presence of some ribosomes and membranous elements. These membranous elements could have been derived from the endoplasmic reticulum or from broken protein bodies. The absence of significant amounts of NADH-cytochrome *c* reductase indicates that the free membranes may originate from protein bodies.

Our earlier studies had shown that α -mannosidase is present in isolated protein bodies (20), and we measured the distribution of this enzyme and of protein on the Ficoll gradients. The results (Fig. 2A) show that more than 75% of the α -mannosidase activity and of the protein was associated with the protein body band. Examination of the protein in the protein bodies by sodium dodecyl sulfate/polyacrylamide gel electrophoresis showed that it consisted almost exclusively of storage protein. The α -mannosidase activity present at the top of the gradient (the load fraction) may represent enzyme released from broken protein bodies. The extent of protein body breakage was determined by analyzing the distribution of reserve protein on the gradients. The load portion of the gradients normally contained 10–25% of the reserve protein (measured as water-insoluble globulin; data not shown) depending on the experiment and the age of the cotyledons. Even after 4 or 5 days of seedling growth 75% of the protein bodies were recovered intact.

Distribution of Hydrolases. To determine if protein bodies contain hydrolases other than α -mannosidase, we measured the distribution of a number of hydrolytic enzymes on the Ficoll gradients. For this experiment we used protoplasts made from cotyledons 36 hr after the start of imbibition. The results, summarized in Table 1, show that 75–80% of the α -mannosidase, *N*-acetyl- β -glucosaminidase, carboxypeptidase, acid phosphatase, phosphodiesterase, ribonuclease, and phospholipase D were associated with the protein body band. However, all the β -amylase was in the supernatant, and the protein bodies did not contain detectable amounts of this enzyme.

Using other methods of isolation, we previously (20) found most of the cellular carboxypeptidase and *N*-acetyl- β -glucosaminidase in the protein bodies. The high levels of acid phosphatase confirm cytochemical experiments indicating that this enzyme is located in the protein bodies (ref. 21, and sum-

FIG. 2. Distribution of α -mannosidase and protein (A) and ribonuclease and proteinase (vicilin peptidohydrolase) (B) on Ficoll gradients of protoplast extracts; cotyledons were obtained from 3½-day-old seedlings. The peak of activity at the 5/25% Ficoll interface represents enzyme associated with the protein bodies. The activity at the top of the gradient represents cytoplasmic enzyme or broken protein bodies. Enzyme activities are expressed in relative units representing a change in absorbance under the conditions used. Protein is expressed as $\mu\text{g}/0.2 \text{ ml}$.

marized in ref. 1). Of particular interest is the localization of phospholipase D. Attempts to localize phospholipase D in plant tissues have been largely unsuccessful, and the function of this enzyme is not understood (see ref. 22 for a review). Incubation of cotyledon extracts results in the rapid degradation of phospholipids with the release of phosphatidic acid, choline, and ethanolamine (16). We have suggested that the action of phospholipase D constitutes the first step in phospholipid breakdown. In cauliflower florets breakdown of phospholipids is catalyzed by a lipolytic acyl hydrolase that is sequestered with other acid hydrolases in lysosome-like organelles (23). The presence of phospholipase D and acid phosphatase in the protein bodies suggests that the rapid catabolism of phospholipids that occurs in the cotyledons (16) may be carried out within the protein bodies.

FIG. 3. (A and B) Electron micrographs of storage parenchyma cells of cotyledons from a $3\frac{1}{2}$ -day-old seedling. In the protein body (PB) shown in A the reserve protein has not yet been digested. Two autophagic vesicles (AV) with cytoplasmic contents are visible. In B most of the reserve protein has been digested and there are two autophagic vesicles, which appear empty. (C and D) Autophagic vesicles in the protein bodies of a $3\frac{1}{2}$ -day-old seedling. (C) The vesicle contains numerous ribosomes, some free, some attached to small vesicles (arrows); (D) autophagic vesicle containing ribosomes and a mitochondrion (M).

These experiments were repeated with protoplasts obtained from cotyledons of $3\frac{1}{2}$ -day-old seedlings, and the results were similar. The distribution of ribonuclease is shown in Fig. 2B. Again it is evident that 80% of the ribonuclease activity is associated with the protein bodies. Our earlier experiments

showed that ribonuclease levels are quite low 24 hr after imbibition and that the enzyme is synthesized during the first 3 days of seedling growth. Our results show that ribonuclease accumulated in the protein bodies after its synthesis.

We also investigated the distribution of proteinase (vicilin

peptidohydrolase) in the cotyledons of 3½-day-old seedlings. This enzyme is absent from the cotyledons at the start of germination and synthesis starts on the third day of seedling growth (6). The results (Fig. 2B) show that some (25%) of the enzyme activity was in the protein bodies and the majority was in the load fraction of the gradients. Later on in seedling growth (4½ days) the majority of the activity was associated with the protein bodies (data not shown). These observations confirm our cytochemical localization of the enzyme by using immunofluorescence (7). On the third day of seedling growth, when the synthesis of the enzyme begins, the enzyme is largely in the cytoplasm, and by the fourth day the enzyme is present in the protein bodies as well as in the cytoplasm.

Electron Microscopy. The presence of acid hydrolases in protein bodies prompted us to look for ultrastructural evidence that protein bodies participate in autophagy and degrade cytoplasmic macromolecules, structures, or both. On the third day of seedling growth, when the digestion of the reserve protein is about to begin, protein bodies contain vesicles (0.2–1 µm in diameter) consisting of a limiting membrane surrounding cytoplasmic structures such as ribosomes (Fig. 3A), elements of the endoplasmic reticulum (Fig. 3C), and occasionally mitochondria (Fig. 3D). At later times, after most of the storage proteins have been digested and the protein bodies appear largely “empty,” the vesicles are also “empty” (Fig. 3B). We interpret these micrographs as follows. Invagination of the protein body membrane causes portions of the cytoplasm to be internalized within the protein bodies, resulting in the formation of the autophagic vesicles shown in Fig. 3. The enzymes present within the protein bodies digest the cytoplasmic structures, leaving an “empty” vesicle. The membranes of the vesicles may be more resistant to enzymatic degradation than the structures contained within, resulting in the presence of “empty” vesicles within the protein bodies that have lost their reserves. The possibility that these vesicles represent cross sections through finger-like cytoplasmic protrusions into the protein bodies was eliminated with serial sections (data not shown). Vesicles similar to those found *in situ* were also observed in isolated protein bodies, confirming the conclusion that they are not cytoplasmic protrusions.

The presence of vesicles in isolated protein bodies opens the way to study the digestion of their contents *in vitro*. This could be done by incubating intact protein bodies or by first isolating the vesicles and determining their enzymatic complement. Our data on the localization of the enzymes show only that the hydrolases are within the protein bodies. How they penetrate into the vesicle through the limiting membrane is not yet clear.

CONCLUSION

The results presented here and elsewhere (6, 7, 20, 24) show that protein bodies of dry and newly imbibed seeds contain certain

acid hydrolases (e.g., α-mannosidase, carboxypeptidase, phosphatase, phosphodiesterase, phospholipase D, and low levels of ribonuclease). In the course of seedling growth new acid hydrolases are synthesized in the cotyledons (especially ribonuclease and vicilin peptidohydrolase), and these accumulate in the protein bodies. The protein bodies therefore constitute the principal lytic compartment in the storage parenchyma cells. We propose that the hydrolases within the protein bodies hydrolyze not only the stored reserves, but also, the cytoplasmic structures and molecules that are internalized by autophagic processes.

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