LOCALIZATION OF VICILIN PEPTIDOHYDROLASE IN THE COTYLEDONS OF MUNG BEAN SEEDLINGS BY IMMUNOFLUORESCENCE MICROSCOPY

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ABSTRACT

Vicilin peptidohydrolase, the protease that hydrolyzes the reserve proteins in the cotyledons of mung bean (*Vigna radiata*) seedlings, has been localized intracellularly by immunofluorescence microscopy using monospecific antibodies against the enzyme and rhodamine-coupled goat-anti-rabbit immunoglobulin G's. The enzyme can first be visualized after 3 days of seedling growth and is associated with small foci within the cytoplasm of the storage parenchyma cells farthest from the vascular bundles. On the 4th day of growth, the protease is also present in the numerous large protein bodies within these cells. Vicilin peptidohydrolase is known to be synthesized *de novo* starting on the 3rd day of growth. Our observations are therefore consistent with the interpretation that the enzyme is synthesized in the cytoplasm and subsequently transported to the protein bodies.

KEY WORDS mung bean · cotyledon · protein body · protease · vicilin peptidohydrolase · immunocytochemistry · immunofluorescence

The cotyledons of leguminous seeds contain abundant reserves of protein which are metabolized in the course of seedling growth. These reserve proteins, called legumin and vicilin, are located in protein bodies, spherical organelles measuring 2–10 μm in diameter and consisting of a dense protein matrix and a limiting membrane (10, 21). Ultrastructural studies show that the dense protein matrix gradually disappears in the course of seedling growth and that the "empty" protein bodies merge to form a central vacuole (16). Such observations indicate that the breakdown of the reserve proteins probably occurs within the protein bodies. Several hypotheses have been advanced to explain how reserve protein catabolism is controlled. It has been suggested that the onset of proteolysis is dependent on the activation of proteases preexisting in the protein bodies (15), on the inactivation of protease inhibitors (18), and on the appearance of new proteases.

Our recent studies, carried out with mung bean seedlings, show that the breakdown of the reserve proteins in the protein bodies is dependent on the *de novo* synthesis of vicilin peptidohydrolase, a sulfhydryl-type endopeptidase (3, 8, 9). This observation indicates that the protein bodies must acquire this enzyme in the course of seedling growth. We tested this hypothesis by isolating protein bodies from cotyledons obtained from seedlings of different ages and found that they contained substantial vicilin peptidohydrolase activity beginning on the 4th day of growth. However, more enzyme activity was present in the

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/78/1001-0010$1.00
Volume 79 October 1978 10-19

supernate of the cotyledon homogenates than in the protein body fraction (9). Using subcellular fractionation, it was impossible to decide unequivocally whether the enzyme present in the supernate originated from broken protein bodies, from the cytosol, or from other broken cytoplasmic organelles. We therefore localized the enzyme by immunofluorescence microscopy. The evidence presented here shows that vicilin peptidohydrolase can first be visualized in small foci in the cytoplasm and accumulates later in the protein bodies.

MATERIALS AND METHODS

Plant Material

Seeds of mung beans (*Vigna radiata* [L] Wilczek, formerly called *Phaseolus aureus* Roxb.) were obtained from a local dealer and grown as previously described (7). For extraction of vicilin peptidohydrolase, the cotyledons were harvested after 5 days of growth and stored at −20°C. For localization experiments, the cotyledons were harvested and immediately used.

Enzyme Purification and Antibody Production

Vicilin peptidohydrolase activity was determined by viscometry as described earlier (2), using 5% gelatin as substrate. One unit of activity represents a decrease in relative viscosity of 1.0 h^{-1}. Protein was measured by the method of Lowry et al. (14), using bovine serum albumin as standard. The enzyme was purified from extracts of cotyledons as previously described (3). The enzyme, judged to be a homogeneous protein preparation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and isoelectrofocusing, was used as antigen. Three rabbits (New Zealand white loop ear hybrids) with an average body weight of 2 kg were each injected subcutaneously with 1 mg of pure enzyme in an emulsion of Freund's complete adjuvant (Calbiochem, San Diego, Calif.). 4 wk later, each rabbit received a second injection of 1 mg of enzyme in Freund's incomplete adjuvant. 7 days later, 30 ml of blood was withdrawn from the ear vein of each rabbit and the serum was removed after coagulation at room temperature. The serum was made 25% (wt/vol) with respect to $(NH_4)_2SO_4$, and the solution was kept at room temperature overnight. The resulting precipitate was sedimented at 4,000 *g* for 30 min and the supernate was discarded. The pellet was washed with 1.75 M $(NH_4)_2SO_4$ and sedimented again. The pellet was dissolved in H_2O and dialyzed overnight against acetate buffer pH 5.0 (0.05 M sodium acetate + 0.021 M acetic acid) in the cold. This solution was passed over a *O*-(diethylaminoethyl)cellulose (DEAE)-Sephadex A-50 column (1.5 × 30 cm) equilibrated with the acetate buffer. The immunoglobulin G fraction that was not adsorbed to the DEAE-cellulose was collected, dialyzed against phosphate-buffered saline (PBS: 0.05 M Na phosphate buffer, pH 7.5, 0.14 M NaCl) and stored with 0.02% sodium azide at 4°C.

Preparation of Monospecific Antibodies

For the immunofluorescence localization experiments, the IgG fraction was made monospecific with respect to vicilin peptidohydrolase. Ultrogel (AcA 22, LKB Instruments, Inc., Rockville, Md.) was activated with glutaraldehyde according to the method of Ternynck and Avrameas (19). To 2 ml of activated gel, 1 mg of pure endopeptidase in 2 ml of 0.1 M phosphate buffer, pH 7.4, was added and the mixture was kept at 37°C while shaking. 92% of the protein bound to the gel, which was then washed with 30 ml of PBS. Then the gel was incubated with 0.1 M lysine in PBS at 37°C for 2 h to mask all the remaining aldehyde groups. The coupled gel was then packed in a small column and washed again with 30 ml of PBS. 2 ml of the IgG fraction containing 8.6 mg of protein was loaded onto the column and kept in the gel for 30 min at room temperature. The column was then eluted with PBS, and fractions of 1 ml were collected. When the absorbance of the eluate at 280 nm dropped below 0.01, the column was washed with 0.2 M HCl-glycine buffer, pH 2.2, and 1-ml fractions were collected into test tubes which were preloaded with 1 M phosphate buffer, pH 7.5, to neutralize the acidic elution buffer as quickly as possible. When the absorbance of the fractions at 280 nm was lower than 0.01, the column was immediately neutralized with PBS. The fractions containing the protein which was adsorbed to the column were combined and the entire procedure was repeated several times. The combined IgG fractions eluted from the column were dialyzed against PBS and concentrated in a minicon B-15 concentrator (Amicon Corp., Lexington, Mass.) to a final concentration of 0.2 mg of protein per ml. This solution containing only monospecific antibodies against vicilin peptidohydrolase was analyzed by double immunodiffusion and immunoelectrophoresis and later used for the immunofluorescence localization of the endopeptidase.

Double Immunodiffusion and Immunoelectrophoresis

Immunodiffusion tests were performed according to Ouchterlony (17) using 1.25% agar (Difco-Nobel) (Difco Laboratories, Detroit, Mich.), 0.15 M NaCl, and 0.001 M EDTA. Diffusion was allowed to proceed for 24 h in a moist chamber.

Immunoelectrophoresis was performed according to the procedures of Grabar and Williams (11). Antigens were electrophoresed in 1% agarose with 0.02 M barbital buffer, pH 8.6, for 120 min at 8–10 V/cm. Incubation with antiserum was performed in a moist chamber for 24 h. After washing and drying of the agarose, the electrophoretograms were stained with Coomassie Brilliant

Blue (ICI United States, Inc., Wilmington, Del.) (5 g of Coomassie Brilliant Blue in 450 ml of ethanol, 100 ml of acetic acid, 450 ml of distilled water) for 10 min and then destained (450 ml of ethanol, 100 ml of acetic acid, 450 ml of H_2O).

Preparation of Rhodamine-Conjugated Goat Antibodies against Rabbit IgG

The conjugation of partially purified goat antirabbit IgG (a gift from Dr. F. Ash of this department) with lissamine rhodamine B (Polysciences, Inc., Warrington, Pa.) was performed following the method of Brandtzaeg (5). 1 g of lissamine rhodamine B was converted to the sulphonylchloride derivative by thoroughly mixing it with 2 g of PCl_5 for 5 min in a mortar at room temperature. After addition of 30 ml of acetone, the slurry was left for 2 min and thereafter filtered through filter paper and kept in a closed bottle at 4°C. To 0.5 ml of goat-anti-rabbit IgG (15 mg/ml) in PBS, 0.5 ml of 1 M bicarbonate-carbonate buffer, pH 9.0, and 50 μl of rhodamine B sulphonyl chloride in acetone was added and the mixture stirred for 30 min on ice. The reaction was stopped by adding 0.5 ml of 1 M NH_4Cl and the mixture was immediately passed over a Sephadex G-50 column (15 × 2 cm) with PBS. The conjugated IgG fraction was then purified by anion-exchange chromatography on DEAE cellulose; selected pools of conjugated IgG fractions were concentrated and dialyzed against PBS according to Bradtzaeg (5) and then passed through a column of Ultrogel AcA 22 to which rabbit IgG (1 mg/ml) had been coupled as described above. The conjugated goat-anti-rabbit IgG which adsorbed to the column was eluted with 0.2 M HCl-glycine buffer, pH 2.2, immediately brought to pH 7.5 with 1 M phosphate buffer, and then concentrated and dialyzed against PBS. This solution, containing selectively rhodamine-labeled goat antibodies immunochemically active against rabbit IgG, was used for the indirect localization of the vicilin peptidohydrolase after appropriate dilution.

Immunofluorescence Localization of Vicilin Peptidohydrolase

Localization of the mung bean endopeptidase was performed by the indirect fluorescent antibody technique. The method described by Bourguignon et al. (4) and Tokuyasu and Singer (20) had to be modified to lead to good results with mung beans. Cotyledons of seedlings grown for 1, 3, and 4 days were harvested, and tissue blocks of ~1 mm^3 were cut directly in 4% paraformaldehyde, 0.1 M sucrose in PBS and fixed for 12 h at 3°C. The tissue was then transferred to 0.9 M sucrose, 4% paraformaldehyde in PBS, and infused with this solution on ice for 5 h. The tissue blocks were mounted on copper rods by freezing in liquid nitrogen, with a film of medium covering the tissue at all times. For cryotomy, the rods with the tissue were transferred to the precooled cryokit bowl of a Sorvall Porter-Blum MT-2B ultramicrotome (DuPont Instruments, Newton, Conn.) at −60°C. Sections ranging from 0.2 to 0.5 μm in thickness were cut with a glass knife at temperatures between −55° and −60°C. The sections were picked up with a small wire loop containing a semifrozen drop of 4% paraformaldehyde, 2.3 M sucrose in PBS. The droplet was brought to room temperature and transferred onto a glass slide which was pretreated with 1% silicone (Siliclad, Clay Adams, Parsippany, N. J.). Glass slides containing six sections were washed for 10 min in an excess volume of PBS and 0.01 M glycine. Small drops of monospecific vicilin peptidohydrolase antibodies (0.2 mg/ml) were then placed over each section and incubated at room temperature in a moist chamber for 8–10 min. The slides were washed again for 10 min in PBS and 0.01 M glycine. Then a droplet of rhodamine-conjugated goat antibodies against rabbit IgG (0.2 mg/ml) was applied to each section for 7 min at room temperature. Afterwards, two washes of 5 min with PBS and glycine were carried out. Subsequently, the sections were mounted in PBS containing 90% glycerol, covered with a cover glass, and examined in a Zeiss Photomicroscope III equipped with Nomarski optics and epifluorescence. Rhodamine was excited with an Osram HBO 50-W bulb (Osram, Berlin, Germany), using a Zeiss rhodamine interference filter. Photography was carried out by using Plux-X film (Eastman Kodak Co., Rochester, N. Y.).

The following controls were always performed simultaneously: sections from 1-day imbibed cotyledons (which contain no vicilin peptidohydrolase) were stained as described; sections from 3- and 4-day-old cotyledons were treated with pre-immune rabbit IgG (0.75 mg/ml) before incubation with rhodamine-labeled antibodies; incubation of the sections with buffer only, to determine possible autofluorescence of the cells; incubation with rhodamine-labeled goat anti-rabbit IgG only.

RESULTS

The Antigen Vicilin Peptidohydrolase

The increase in endopeptidase activity in the cotyledons of germinating mung beans is shown in Table I. Almost no enzyme activity could be measured in extracts of dry beans. The activity increased slowly during the first 3 days of growth and increased very rapidly during the 4th and 5th days of seedling growth. During the last period of germination between day 6 and day 8, the endopeptidase activity decreased again. Enzyme from 5-day-old cotyledons was used as antigen. Vicilin peptidohydrolase was purified as described (3) and was judged to be homogeneous by SDS-polyacrylamide gel electrophoresis and isoelectrofocusing (3).

TABLE I

Changes in Endopeptidase Activity in the Cotyledons of Mung Beans during the First 8 Days of Seedling Growth

Days of seedling growth	Endopeptidase activity
	*units/bean**
0	0.051‡
1	0.091‡
2	0.32
3	0.61
4	2.45
5	3.55
6	2.93
7	1.89
8	1.55

Cotyledons were collected at 1-day intervals, and 40 cotyledons were homogenized in 10 ml of buffer and centrifuged at 20,000 *g* for 20 min. Endopeptidase activity was determined on the clear supernate by viscometry.

* A decrease in relative viscosity of 1.0 represents 1 unit of enzyme activity.

‡ Not due to vicilin peptidohydrolase activity (7).

Specificity of the Antiserum and the Purified Antibodies

The purity of the antigen vicilin peptidohydrolase was demonstrated by double immunodiffusion. A single precipitin arc was formed when a crude extract of cotyledons from 5-day-old seedlings was allowed to diffuse against the antiserum. No precipitin band was formed with pre-immune serum or extracts of cotyledons from 1-day-old seedlings (data not shown; see reference 8). The purity of the antigen preparation was confirmed by immunoelectrophoresis. Again, only a single precipitin arc formed after the proteins from a crude extract of 5-day-old cotyledons were fractionated by electrophoresis (Fig. 1).

To obtain monospecific antibodies against vicilin peptidohydrolase, the IgG fraction of the antiserum was fractionated by affinity chromatography. When this fraction was passed over a column consisting of Sepharose covalently linked to purified vicilin peptidohydrolase, most of the protein was eluted with the loading buffer. A small fraction (2%) of the initial protein was retained and could be eluted as a single peak with the acidic buffer (Fig. 2). The monospecific antibodies obtained by this method showed the same immunological specificity as the complete serum.

Localization of Vicilin Peptidohydrolase

The cotyledons of mung bean seedlings contain different cell types. Most of the tissue consists of large storage parenchyma cells containing starch grains and numerous protein bodies. The cotyledons are traversed by vascular bundles which are readily visible in cross sections as little islands of 5–10 small cells (11). Under the conditions of fixation and preparation used here, the storage parenchyma cells showed no autofluorescence

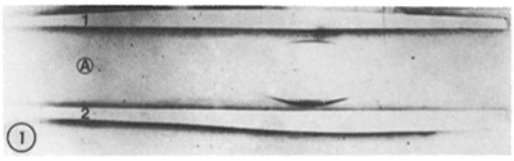

FIGURE 1 Precipitin reactions in agar gel after electrophoresis and diffusion. A crude homogenate from 5-day-old cotyledons was placed in the middle well (*A*) and electrophoresed in 1% agarose at 10 mA for 120 min and then allowed to diffuse overnight in a moist chamber against whole anti-vicilin peptidohydrolase serum (trough *1*) and monospecific antibodies against vicilin peptidohydrolase (0.10 mg/ml) in trough *2*. Hence, whether the whole antiserum or the purified monospecific antibodies were allowed to react against the supernate of a homogenate from 5-day-old cotyledons, similar single precipitin bands were obtained.

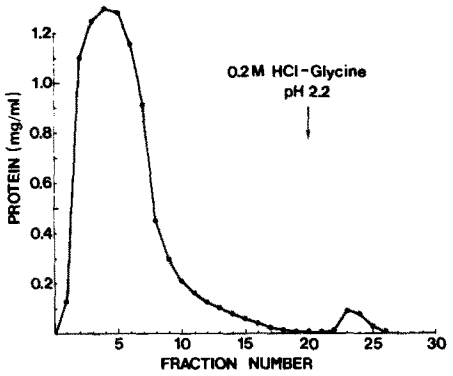

FIGURE 2 Affinity chromatography of the rabbit IgG fraction containing antibodies against vicilin peptidohydrolase on an antigen column. Pure vicilin peptidohydrolase was covalently linked to Ultragel AcA 22 (0.5 mg protein/ml packed gel) and the gel packed into a small column. The IgG-fraction (4.33 mg protein/ml) from the rabbit serum was passed through the column which was equilibrated with PBS. Antibodies that bound to the ligand were eluted with 0.2 M HCl-glycine buffer, pH 2.2.

whereas the cell walls of some of the cells in the vascular bundles exhibited autofluorescence. To distinguish between specific and nonspecific staining, the following controls were routinely made. Sections originating from the same tissue were incubated with pre-immune (non-immune) serum followed by incubation with rhodamine-labeled antibodies, or incubation of the sections with rhodamine-labeled antibodies alone. As a result of the high purity of the antibodies and the short incubation times used in these experiments, none of these control experiments yielded any nonspecific background fluorescence. As we showed earlier (9), 1-day imbibed cotyledons contain no vicilin peptidohydrolase; therefore, sections from such cotyledons could serve as additional tissue controls. When sections from 1-day-old cotyledons were stained for endopeptidase, no fluorescence was observed (Fig. 3).

When thin sections obtained from 3-day-old cotyledons were treated with monospecific antibodies against vicilin peptidohydrolase, strong fluorescence appeared in small foci throughout the cytoplasm of certain cells (Fig. 4). Fluorescence first appeared in cells farthest from the vascular bundles. No fluorescence was observed in the protein bodies, in the cell walls, or in extracellular spaces. When the same treatment was applied to sections from 4-day-old cotyledons, fluorescence was observed in all the protein bodies of a particular cell (Fig. 5). At the same time, fluorescence was still associated with the small foci within the cytoplasm as observed in cotyledons from 3-day-old seedlings. Neighboring cells were found to be at the 3-day stage, and vicilin peptidohydrolase was confined to small foci in the cytoplasm, or in a physiological older stage. In these older cells, the protein bodies had lost their spherical shape, and proper fixation and staining was not possible with the method described.

DISCUSSION

Although several hypotheses have been advanced to explain how reserve protein catabolism is regulated in legume seedlings, our recent experiments with mung beans strongly support the interpretation that the onset of reserve protein breakdown is dependent on the *de novo* synthesis of a new endopeptidase (9). We purified this enzyme, demonstrated that it is capable of hydrolyzing vicilin, the major reserve protein of mung beans, and called the enzyme vicilin peptidohydrolase (3). Ultrastructural evidence obtained by Öpik (16) indicates that reserve protein hydrolysis probably occurs within the protein bodies. This interpretation is strengthened by our observation that the cytosol contains a potent inhibitor of vicilin peptidohydrolase (2). The demonstration that vicilin peptidohydrolase is localized in the protein bodies would strengthen our interpretation that this enzyme, which accounts for >95% of the endopeptidase activity in the cotyledons, plays a major role in reserve protein catabolism. Our attempts to localize the enzyme by fractionation of the subcellular organelles on sucrose gradients did not lead to unequivocal results (9). Starting on the 4th day of growth, endopeptidase activity was present in the protein body fraction but also in the supernate. We therefore decided to localize the enzyme by immunofluorescence microscopy. Endogenous fluorescence was only found in the walls of small cells of the vascular bundles and resulted most likely from the fluorescence of lignin present in these walls. Nonspecific fluorescence was a major problem before the use of affinity purified IgG preparations. Nonspecific sticking of the antibodies to the sections (resulting in nonspecific fluorescence) was eliminated by the use of monospecific antibodies against vicilin peptidohydrolase. This nonspecific sticking which occurs at higher IgG concentrations may be the result of the abundant presence of lectins in the cotyledons. To achieve a low background staining with rhodamine-labeled goat-anti-rabbit IgG, we used only IgG fractions with selected molar ratios of fluorochrome to protein as discussed by Brandtzaeg (5) and purified them further on an affinity column consisting of rabbit IgG covalently linked to Sepharose. Application of these extensively purified IgG fractions to thin sections (0.2–0.5 μm) resulted in negligible nonspecific staining. The antigenicity of the vicilin peptidohydrolase was preserved by the relatively mild fixation with formaldehyde and the infusion with sucrose before freezing and sectioning with the cryostat. Antibodies have greater access to their antigens in frozen-section tissue than in embedded tissue.

The results reported here show that the cotyledons contain no vicilin peptidohydrolase activity after 1 day of growth, confirming our earlier observation that no immunologically cross-reacting material was present in extracts of cotyledons until the third day of growth (9). Fluorescence caused by the presence of vicilin peptidohydrolase can first be observed on the 3rd day of growth. The antigen appears to be present in small foci in

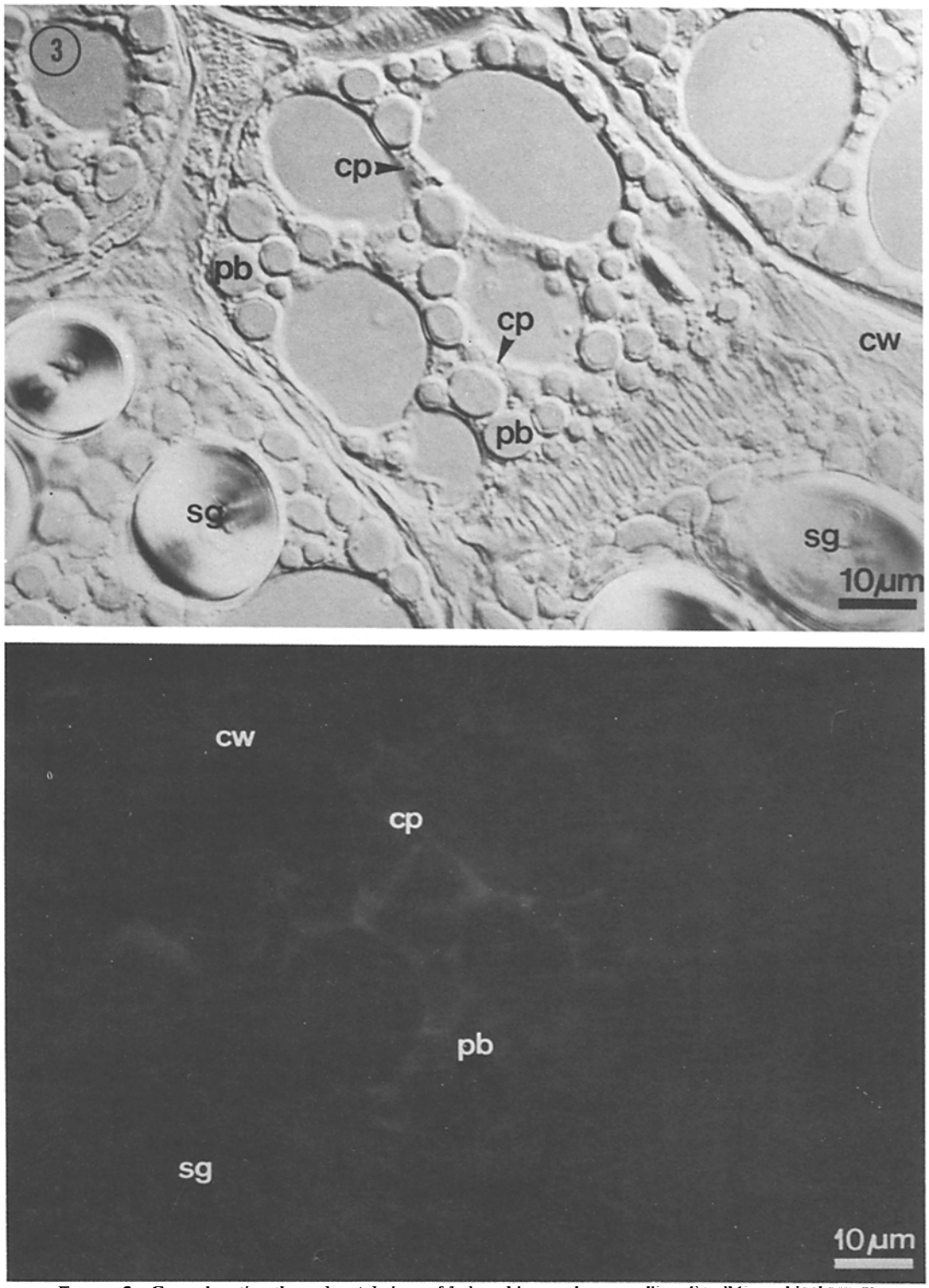

FIGURE 3 Control section through cotyledons of 1-day-old mung bean seedling. Small tissue blocks were fixed in 4% paraformaldehyde, 0.1 M sucrose in PBS for 12 h at 3°C. The tissue was infused with 0.9 M sucrose and then frozen in liquid nitrogen. Frozen sections of 0.5 μm in thickness were cut and placed on glass slides where they were incubated with monospecific anti-vicilin peptidohydrolase antibodies followed by staining with rhodamine-labeled goat antibodies against rabbit IgG. (*Top*) Photomicrograph taken with Nomarski optics. Note the many large protein bodies (*pb*), the cell wall (*cw*), the cytoplasm (*cp*), and the starch grains (*sg*). The large open areas originate from starch grains lost during sectioning. (*Bottom*) Photomicrograph of the same cell photographed with epifluorescence. As 1-day-old cotyledons contain no vicilin peptidohydrolase, no specific fluorescence can be observed. Only a very faint background fluorescence is associated with the cytoplasm. × 1,200.

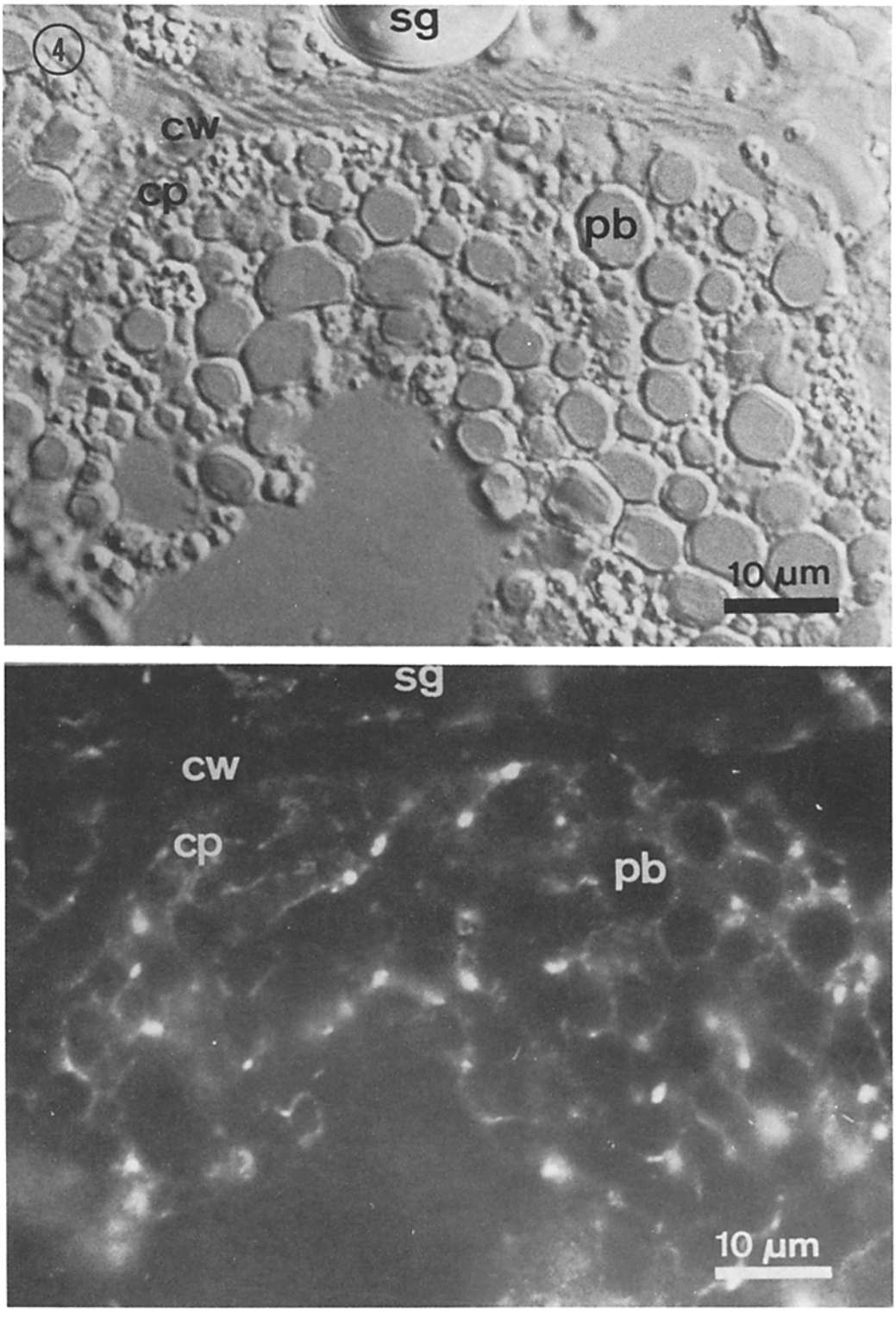

FIGURE 4 Localization of vicilin peptidohydrolase by immunofluorescence microscopy in cotyledons from 3-day-old seedlings. (*Top*) A section (0.5 μm) photographed with Nomarski optics. Cell walls (*cw*), protein bodies (*pb*), cytoplasm (*cp*), starch grains (*sg*). (*Bottom*) Same cell photographed with epifluorescence. Light areas represent staining with fluorescent antibodies. Note that the stain is present in distinct foci in the cytoplasm but not in the protein bodies and the cell walls. × 1,700.

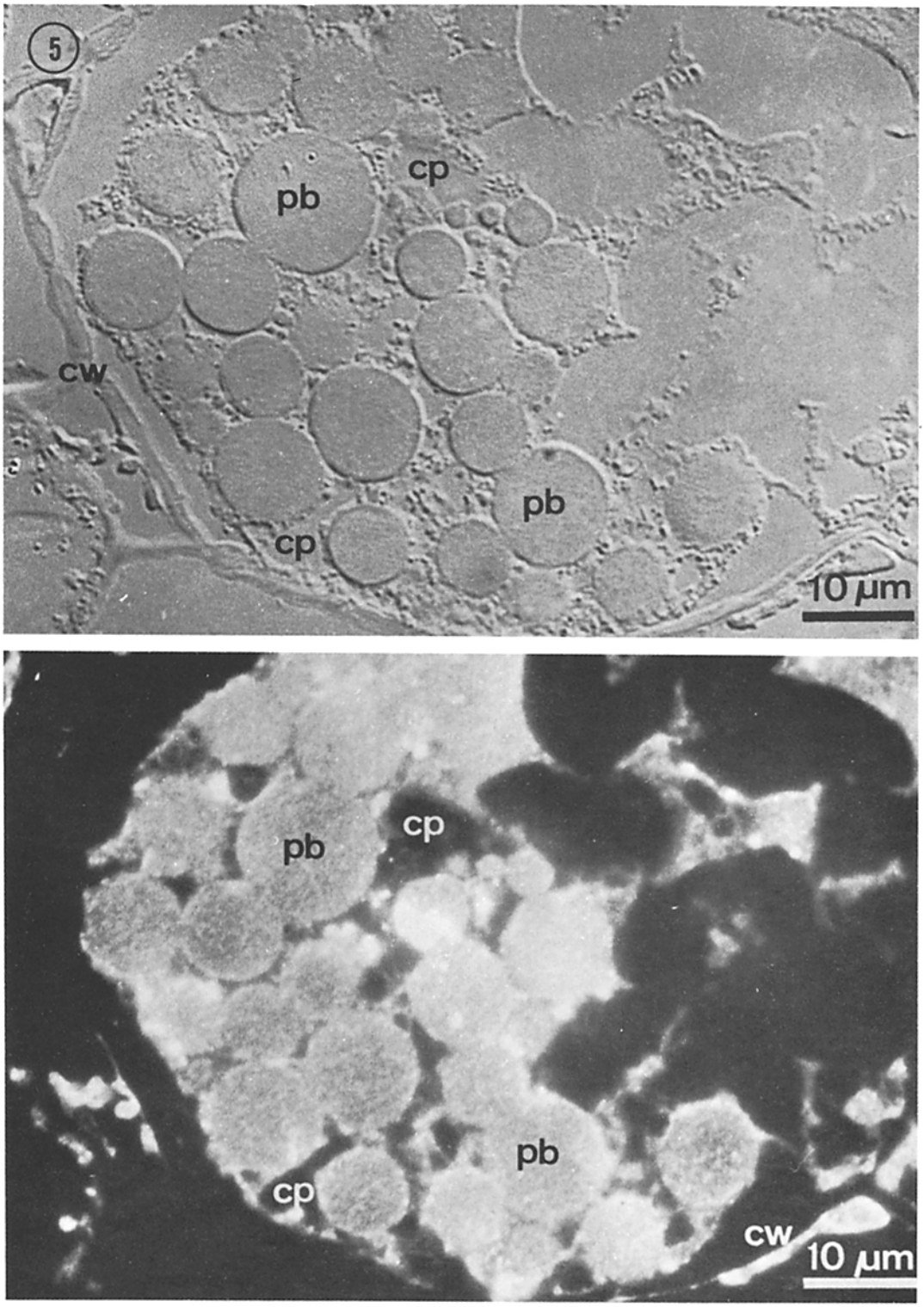

FIGURE 5 Localization of vicilin peptidohydrolase by immunofluorescence microscopy in a 0.2-µm thick section from a 4-day-old cotyledon. (*Top*) A cell photographed with Nomarski optics; cell walls (*cw*), protein bodies (*pb*), cytoplasm (*cp*). (*Bottom*) Photomicrograph of the same cell taken with epifluorescence. Fluorescent antibodies are still associated with distinct areas in the cytoplasm as in 3-day-old cotyledons (Fig. 4), but in addition the protein bodies (*pb*) are stained with anti-vicilin peptidohydrolase antibodies. × 1,700.

the cytoplasm. This distribution contrasts with that of trypsin inhibitor which we localized with the same method (7). Fluorescence due to the presence of trypsin inhibitor is uniformly distributed over the cytoplasm, and there are no apparent foci of activity as shown here for vicilin peptidohydrolase.

Fluorescence is found first in the cells farthest from the vascular bundles, confirming earlier cytochemical work (12, 16, 22) showing that protein breakdown begins in the cells farthest from the vascular bundles. The small foci probably represent either sites of enzyme synthesis (cisternae of rough endoplasmic reticulum?) or sites of enzyme accumulation (dictyosomes? transport vesicles?). Positive identification of the foci will require the use of immunocytochemical methods at the ultrastructural level. On the 4th day of growth, most of the fluorescence is over the protein bodies while fluorescence is still present in the small cytoplasmic foci. The experiments lack a dynamic dimension, but the results are consistent with the interpretation that the enzyme is synthesized in the cytoplasm and transported to the protein bodies. Indeed, protein bodies of legume cotyledons, unlike the protein bodies of corn endosperm (13), have no ribosomes attached to their limiting membrane. As a result, it is necessary to transport enzymes which accumulate in protein bodies from their site of synthesis in the cytoplasm to these protein bodies.

Ultrastructural observations show that the cisternae of the rough endoplasmic reticulum proliferate during the first 3–4 days of growth (12, 16) and that this proliferation is accompanied by a 20-fold increase in the endoplasmic reticulum (ER) marker enzyme NADH-cytochrome c reductase (M. J. Chrispeels. Unpublished observation). This new rough ER may well be the site of synthesis of the vicilin peptidohydrolase. The proliferation of rough ER in the cotyledons is accompanied by the appearance of vesicles in the cytoplasm which merge with (or bleb off from) the protein body membrane (8). These vesicles could be the transport vehicles for the vicilin peptidohydrolase and represent the small foci reported here. There is other evidence that indicates that proteins which accumulate in the protein bodies are synthesized by the rough ER. During seed maturation, when protein bodies are being formed in the developing cotyledons, reserve proteins are synthesized by the rough ER before their sequestration in the protein bodies (1).

Unfortunately, it is not possible to follow the further destiny of the endopeptidase after the reserve proteins have been digested. Without the reserve proteins as matrix, the "empty" protein bodies could not be preserved through the fixation and sectioning procedure. In physiologically older cells, vicilin peptidohydrolase was found throughout the cytosol probably as a result of the rupture of the protein bodies.

Our finding that vicilin peptidohydrolase is located in the protein bodies supports the hypothesis, elaborated elsewhere (6), that the synthesis of this enzyme is the critical factor in the control of reserve protein breakdown in the cotyledons of mung bean seedlings. Once the enzyme has been added to the protein bodies, breakdown of reserve protein can begin and is the result of the cooperative action of endopeptidase and carboxypeptidase (3).

It is a pleasure to acknowledge the helpful advice from Dr. J. Slot. Some of the experiments were carried out in the laboratories of Dr. S. J. Singer where work is supported by grants from the U. S. Public Health Service.

Financial support for this work came from the National Science Foundation (a grant to Dr. Chrispeels) and the Swiss National Science Foundation (a fellowship to Dr. Baumgartner).

Received for publication 23 January 1978, and in revised form 1 June 1978.

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