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Imunohistochemical Localization of α-Amylase in Cotyledons of *Vigna mungo* Seedlings[1]

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ABSTRACT

We studied the localization of α-amylase with indirect fluorescence microscopy in transversely sectioned cotyledons of *Vigna mungo* seedlings. Tissue sections were fixed in periodate-lysine-paraformaldehyde and treated with anti-α-amylase immunoglobulin G followed by fluorescein isothiocyanate labeled goat anti-rabbit immunoglobulin G. α-Amylase appeared in the cells farthest from vascular bundles on the second day of growth and appeared gradually closer to the vascular bundles as growth progressed. The pattern of α-amylase appearance was similar in detached cotyledons, indicating that attachment of the embryonic axis has no effect on this pattern. However, in attached cotyledons, α-amylase disappeared from the regions where starch grains had been digested, but in detached cotyledons there was no disappearance of α-amylase, and digestion was slower than in intact cotyledons.

When legume seedlings grow, the starch grains in cotyledonary cells are digested and the resulting sugars used for axis growth. There is not much information about the developmental regulation of α-amylase synthesis in cotyledons of legume seedlings, especially at tissue levels (1). *Vigna mungo* is an exalbuminous legume, in which the cotyledonary cells are filled with starch grains as the main energy storage component. During seedling growth, the amount and the activity of α-amylase in intact cotyledons increases until the 5th d after imbibition and then decreases; in detached cotyledons, on the other hand, the level of α-amylase increases continuously more than 3 times the maximum level in intact ones throughout a 6 d incubation period (5). Zymograms of activities and Ouchterlony double immunodiffusion test on the activities of attached and detached cotyledons showed that the increase of α-amylase in detached cotyledons was due to the identical enzyme as in attached tissue (5). Our previous histochemical observations (8) of germinating *V. mungo* seeds using the starch gel film method showed that the α-amylase activity in intact cotyledons increased in the cells farthest from the vascular bundles. However, in detached cotyledons the localization of the enzyme was unclear. In the present study, we have used immunohistochemical techniques to clarify whether the developmental patterns of α-amylase are really different in attached and detached cotyledons. Furthermore, we discuss the possibility that there is a specialized tissue where α-amylase is synthesized and secreted to other cells of the *V. mungo* cotyledon.

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MATERIALS AND METHODS

Plant Material. *Vigna mungo* seeds were germinated and seedlings were grown at 27°C. Detached cotyledons from the seeds were incubated at 27°C in the dark as described (5), except that surface sterilization of detached cotyledons was performed in 1% NaOCl solution for 5 min.

Purification of α-Amylase and Anti-α-amylase Immunoglobulin G. α-Amylase was isolated from 4-d-old cotyledons of *V. mungo* seeds and purified using affinity chromatography on β-cyclodextrin Sepharose as described (4). Rabbit antiserum against α-amylase obtained previously (5) was purified further by chromatography on an α-amylase-linked Sepharose-4B column. The immunosorbent column was prepared by coupling 2 mg of purified α-amylase with 1 ml cyanogen bromide-activated Sepharose-4B. Anti-α-amylase serum was applied to the column equilibrated with 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. After washing out with PBS[2] all of the γ-globulin not bound to the column, the anti-α-amylase IgG was eluted by the same buffer containing 1 mM NaOH. The eluate was immediately neutralized with 10 mM HCl and stored at −20°C until use. The specificity of the purified IgG to the α-amylase was tested by immunoelectroblotting using materials from Bio-Rad according to the procedure described by the manufacturer. Purified α-amylase and proteins extracted with SDS sample buffer (made of 50 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 0.002% bromphenol blue, and 20% glycerol) from intact or detached cotyledons at various stages were separated by electrophoresis using 12.5% SDS-polyacrylamide slab gel with 1 mm thickness (6). After electrophoretic separation, the proteins in the gel were transferred to nitrocellulose paper in buffer (5 mM Tris, 35 mM glycine, and 6 M urea) for 2 h at 125 V. The nitrocellulose paper was immersed in blocking solution (10% fetal calf serum) for 30 min and then reacted with a purified anti-α-amylase IgG (0.02 mg/ml) for 1 h. After removal of free and nonspecifically bound antibody by washing the paper twice in Tween-containing buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.05% Tween-20) for 10 min each; the paper was transferred to the second antibody solution (horseradish peroxidase conjugated goat-anti-rabbit IgG, used 2500 times diluted) for 1 h. The paper was washed with Tween-containing buffer and then immersed in horseradish peroxidase color development solution.

Immunofluorescent Microscopic Experiments. The indirect immunofluorescence technique employed was similar to that described by Okamoto and Akazawa (10). At various stages, the intact or detached cotyledons were harvested and stored at −80°C. After embedding in Tissue Tek II (Miles) and freezing in liquid N_2, the cotyledons were sectioned transversely at 20 μm thickness with a Cryostat (Lipshow). The sections were air-dried

[2] Abbreviations: PBS, sodium phosphate buffer saline; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate.

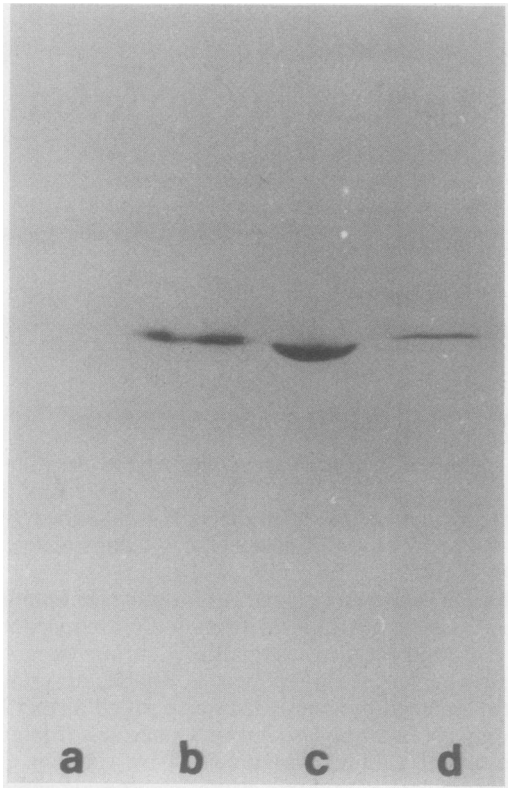

FIG. 1. Immunoelectroblotting of crude extract of *V. mungo* cotyledons with anti-α-amylase rabbit IgG. (a), Six h after imbibition; (b), 4 d after imbibition; (c), detached cotyledon incubated for 5 d; (d), purified α-amylase.

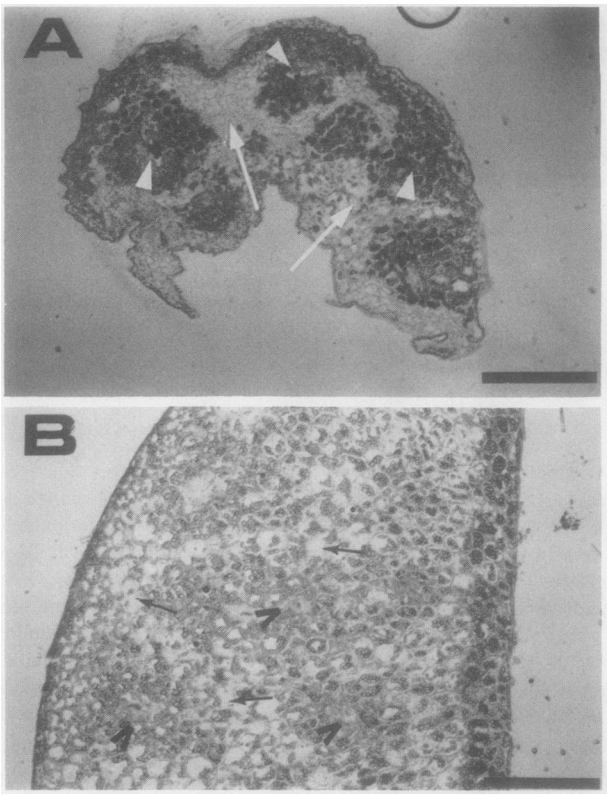

FIG. 2. Distribution of starch reserves in cotyledon sections of *V. mungo*. Transverse sections were cut 20 μm thickness from cotyledon of 4-d-old seedlings (A) or detached cotyledons incubated for 5 d (B). The size bars represent 500 μm. The positions of vascular bundles are marked by arrowheads.

on cover slips which had been evenly coated with a thin film of 2% gelatin (w/v). The sections were fixed with methanol for 10 min to extract lipids and were treated with periodate (10 mM periodate, 50 mM lysine, 2% paraformaldehyde) for 15 min. After washing 3 times (15 min each) in PBS, the sections were incubated in 10% fetal calf serum at 26 to 28°C for 30 min in a moist chamber to avoid nonspecific binding between tissue and first and second antibody serum. The sections were washed 3 times (15 min each) in PBS and incubated with the purified rabbit anti-α-amylase IgG (0.1–0.2 mg/ml) at 27°C for 40 min (in a moist chamber), and washed 3 times (15 min each) in PBS. The sections were then incubated with FITC-conjugated goat anti-rabbit IgG (Miles, F/P = 2.7) at a 50-fold dilution, pretreated with the acetone powder of 1-d germinated *V. mungo* cotyledons for 40 min to avoid nonspecific binding to the tissue, and washed 3 times (15 min each) in PBS. The specimens were briefly rinsed in distilled H_2O to avoid salt crystallization and cover slips were mounted over 90% glycerol (w/v) in 0.5 M carbonate buffer, pH 9.5. The sections were examined under an Olympus fluorescence microscope. The following controls were routinely employed: (a) phosphate-buffered saline only; (b) treatments with anti-α-amylase IgG were omitted; and (c) nonimmune rabbit serum was used instead of anti-α-amylase IgG.

RESULTS

We have previously demonstrated (5) the specificity against α-amylase of our antiamylase serum using both Ouchterlony double immunodiffusion and immunoprecipitation. To examine the possibility of nonspecific binding of proteins of *Vigna mungo* cotyledons with the purified anti-α-amylase IgG, an immunoelectro-blotting test was carried out using SDS soluble proteins extracted from the tissues at several stages of seedling growth. Only a single protein band was detected on nitrocellulose paper when the crude extracts of 4-d-old intact (lane b) and 5-d-old detached (lane c) cotyledons were electrophoresed and immunoreacted (Fig. 1). These protein bands correspond to that of the purified α-amylase (lane d). α-Amylase was not detected in the cotyledons 6 h after imbibition (lane a). Thus, it is evident that the purified antibody is monospecific toward α-amylase of *V. mungo* cotyledons and also that the α-amylase is synthesized *de novo* after the onset of germination.

The degradation of reserves, mainly starch grains, starts in tissue areas farthest from the vascular bundles in intact cotyledons. When the cellular reserves appear to be almost totally exhausted, the cells collapse, and as a result the cotyledons shrivel (Fig. 2A). In detached cotyledons, a decrease of cellular inclusions is also observed, but the process is much slower; the cells become vacuolated and starch grains are pushed to the periphery of each cell. Thus, the size of the detached cotyledon increases gradually during the period of 3 to 5 d of incubation (Fig. 2B).

The results of the immunofluorescence labeling of sectioned cotyledons treated with anti-α-amylase IgG followed by conjugation with the FITC-labeled goat anti-rabbit IgG are shown in Figure 3. In the attached cotyledons, weak fluorescence of FITC is detected farthest from vascular bundles (arrowheads) in 2-d-old cotyledon (Fig. 3A). In sections from 3-d-old and 4-d-old cotyledons (Fig. 3, B and C), the fluorescent area appeared gradually close to vascular bundles (arrowheads). However, fluorescence initially detected farthest from the vascular bundles is not found any longer in 4-d-old specimens, where starch is almost completely exhausted (Figs. 2A and 3C, arrows). In the detached cotyledons, we found that the distribution of fluorescence was similar to that in the attached cotyledons (Fig. 3, E and F). In contrast to the attached cotyledons, however, as shown in the

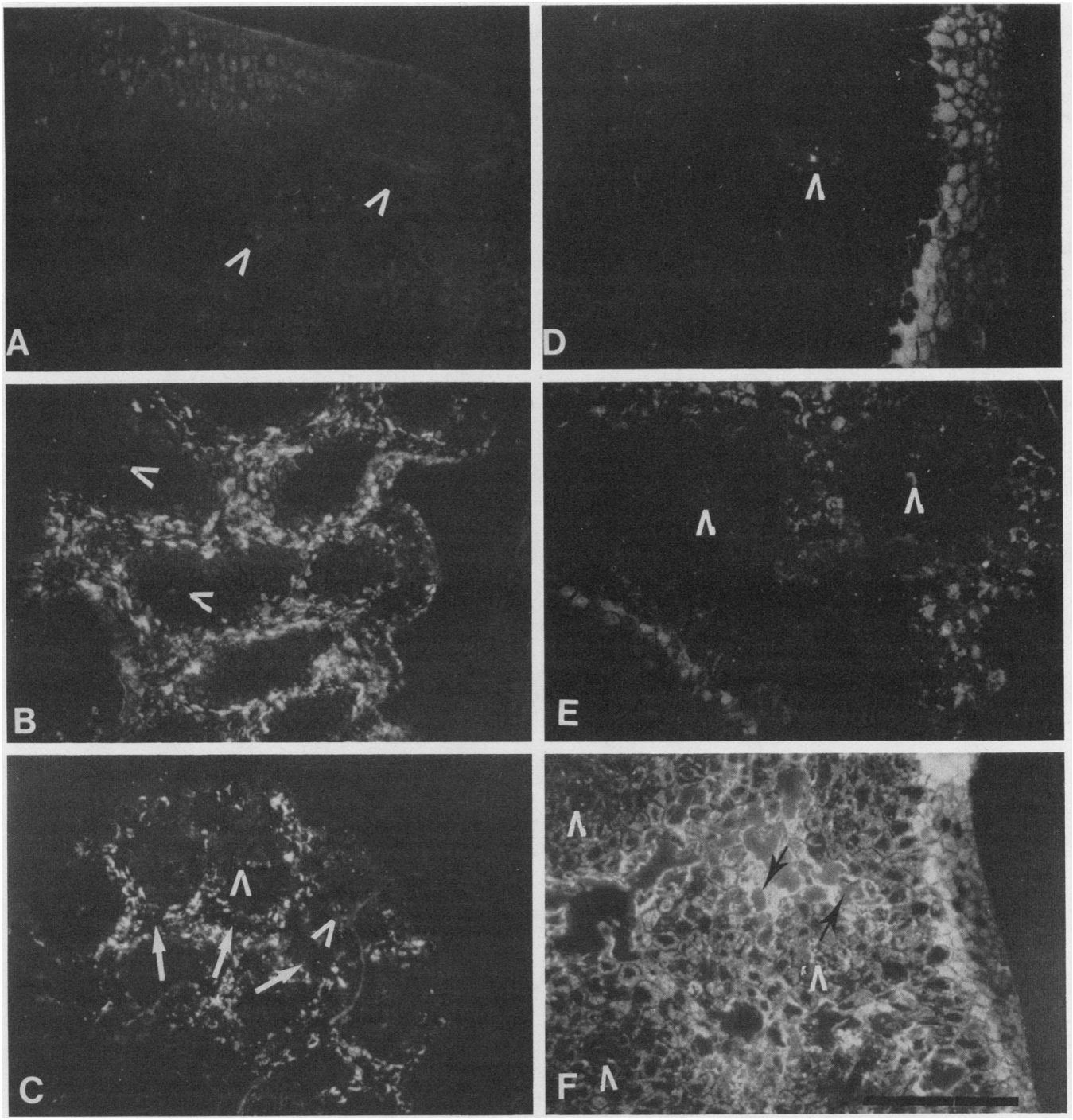

FIG. 3. A to F, Immunofluorescence localization of α-amylase in thin sections of *V. mungo* cotyledons. Transverse sections of cotyledons at different stages: A, B, and C attached cotyledons of seedlings which were 2, 3, and 4 d old, respectively; E and F detached cotyledons incubated for 3 and 5 d, respectively; D, detached cotyledon incubated for 3 d and treated with nonimmune rabbit serum plus FITC-conjugated goat anti-rabbit IgG. Regions from which immunofluorescence disappears are marked by arrows. Distribution of vascular bundles is marked by arrowheads. Size bar = 500 μm.

case of the d-5 specimen (Fig. 3F), fluorescence did not disappear from the areas where it was initially detected, even where starch grains were exhausted almost completely (Figs. 2B and 3F, arrows). As one example of a control, Figure 3D shows a detached 3-d-old cotyledon which was treated with nonimmune rabbit serum instead of α-amylase IgG; autofluorescence was observed at the vascular bundles (arrowheads) and in damaged areas. Other controls (see "Materials and Methods") gave similar results (data not shown).

DISCUSSION

It is well known that in cereal seeds gibberellin produced in the embryo moves to the aleurone layer where it stimulates α-amylase synthesis and secretion into the starchy endosperm (1, 3). Comparing the relation between embryonic axis and cotyledon with embryo and endosperm in cereal seeds, many investigations have been performed including exogenous hormonal treatments in exalbuminous legume seeds (1, 7, 12, 13). How-

ever, a clear account has not been obtained with regard to the relationship between the axis and the cotyledon. At the tissue level, the degradation of food reserves such as protein and starch in legume cotyledons was investigated in detail (2, 11), but there have been only a few investigations on the appearance of hydrolytic enzymes at the cellular level (2, 8, 14).

We previously reported histochemical observations with cotyledons of *Vigna mungo* seedlings, showing that amylolytic activity initially increased in tissue areas farthest from vascular bundles; the mobilization of starch reserves also started in these areas. In the present study, α-amylase in intact cotyledon was also initially detected in tissue areas farthest from vascular bundles and then appeared closer to the vascular bundles as seedling growth progressed. The appearance of α-amylase was not altered when the embryonic axis was removed from the cotyledons, indicating that the appearance of α-amylase has already been determined during the seed maturation period. As shown in Figure 3C, however, α-amylase could not be detected in the areas where it initially appeared. This result indicates that there are no specialized cells where the α-amylase is synthesized and transported to other regions. So, in the cotyledons of *V. mungo* seeds, α-amylase is probably synthesized in individual cells closer and closer to the vascular bundles.

Our immunohistochemical observations agree with previous results showing that the α-amylase content in detached cotyledons increased continuously and reached a higher level than in attached cotyledons (5). In detached cotyledons, α-amylase did not disappear from tissue areas where it initially appeared. This may be due to the suppression of proteinases in the cotyledons when the embryonic axis is removed (9).

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