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Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds

8. IMMUNOHISTOCHEMICAL LOCALIZATION OF β-AMYLASE

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ABSTRACT

Rabbit antiserum against β-amylase isolated from germinating seeds of rice was produced, and its specific cross-reactivity with β-amylase was confirmed by means of Ouchterlony double immunodiffusion and immunoelectrophoresis procedures. The cellular localization of β-amylase was studied by indirect fluorescence microscopy of thin sectioned germinating rice seed specimens (1-day stage) which had been fixed and treated with purified rabbit anti-β-amylase immunoglobulin G followed by conjugation with fluorescein isothiocyanate-labeled goat antirabbit immunoglobulin G. It has been demonstrated that β-amylase is uniformly associated with the periphery of starch granules in the starchy endosperm cells. The finding is discussed in relation to the general notion concerning the presence of the latent form of β-amylase bound to protein bodies in cereal seeds.

There exist two types of amylase in the plant world, α-(EC 3.2.1.1) and β-(EC 3.2.1.2) amylase. How these two hydrolases, differing in their mode of action, are involved in the breakdown of the reserve starch molecules is still a mystery. Although there is no direct evidence to show that β-amylase is truly involved in the starch hydrolysis during germination of starchy seeds, the coordinated interaction of α- and β-amylase in the complete breakdown of starch has been postulated (21). It has been claimed that β-amylase, unlike α-amylase, cannot attack the native starch granules (27); based on experimental results of model experiments, it has been hypothesized that β-amylase is involved in the secondary step of starch hydrolysis after the initial action by α-amylase (7). It is generally agreed that the inactive latent form of β-amylase molecules associated with other proteins in cereal seeds can be cleaved either enzymically or by reducing agents, and eventually an active enzyme is released (23, 24, 26). This general picture is in sharp contrast to that of α-amylase which is synthesized following the triggering action of GA[1] (5, 9).

Here, we have attempted to explore the cellular localization of β-amylase in rice seeds at the early germination stage by the immunochemical technique. Rabbit antiserum against purified β-amylase from germinating rice seeds was prepared and indirect immunofluorescence microscope examination was employed using the IgG fraction of the antiserum to demonstrate the association of β-amylase with starch granules.

[1] Abbreviations: FITC: fluorescein isothiocyanate; GA: gibberellin (gibberellic acid); IgG: immunoglobulin G; PLP: periodate-lysine-paraformaldehyde solution.

MATERIALS AND METHODS

Purification of β-Amylase and Development of Rabbit Anti-β-Amylase Serum. β-Amylase, isolated from germinating rice (*Oryza sativa* L. cv. Kimmazé) seed extracts, was purified essentially following the method reported previously (19). Homogeneous preparation of β-amylase (band C) as revealed by polyacrylamide gel isoelectric focusing (pH 4–6) was used for the immunization of a rabbit. The injection of the antigen into the rabbit was carried out at weekly intervals in four injections, each containing 1 mg of the pure β-amylase sample mixed with the complete Freund's adjuvant in the first injection; the subsequent three injections were mixed with the incomplete adjuvant.

Purification of Anti-β-Amylase IgG. Rabbit antiserum against β-amylase was purified to the γ-globulin fraction by 50% $(NH_4)_2SO_4$ (w/v) fractionation and subsequent DEAE-cellulose column chromatography (22). The monospecific antibody against β-amylase was isolated by passage through a column of β-amylase-bound Sepharose 4B (see Fig. 2). The immunosorbent Sepharose 4B column was prepared by coupling 0.5 mg purified β-amylase with 0.5 g CNBr-activated Sepharose 4B according to the procedure described in the brochure supplied by the manufacturer (Pharmacia, Uppsala). Then the γ-globulin fraction of anti-β-amylase serum, which was previously dialyzed against 0.02 M borate buffer (pH 8.0) containing 0.14 M NaCl, was applied to a column. After all of the γ-globulin not bound to the immunosorbent column was eluted with the above borate buffer, the elution buffer was changed to 0.2 M glycine-HCl buffer (pH 2.8) to liberate the bound anti-β-amylase IgG. The IgG fraction thus eluted was concentrated after neutralizing with 1 M Na-phosphate.

Ouchterlony Double Immunodiffusion. The specificity of the antiserum prepared was tested by means of the Ouchterlony double immunodiffusion agar plate, which was carried out according to a method we reported previously (18). Cross-reactions were tested by various antigen-antibody combinations, placing the antiserum in the center well and antigenic proteins in outer wells. Incubation temperature was 20 C.

Immunoelectrophoresis. Immunoelectrophoresis was performed according to the procedure of Scheidegger (25). Antigenic protein samples were electrophoresed in 50 mM barbital-acetate buffer (pH 8.6) with a constant current of 15 amp (4 C for 2 h). Duration of incubation with antibody placed in the trench varied to give the optimal precipitin formation. Incubation temperature was 20 C. For both the Ouchterlony immunodiffusion and the immunoelectrophoresis experiments, crude extracts of rice seed endosperm (7-day germination stage) were prepared following the procedure reported previously (19).

Immunofluorescence Microscope Experiment. The indirect immunofluorescence technique employed was basically similar to that described by Jacobsen and Knox (12). One-day germinated

rice seeds were cut in half and fixed at 4 C for 12 h in (a) 4% paraformaldehyde (w/v) dissolved in 20 mM phosphate-buffered saline (pH 7.4) or (b) 0.01 M periodate-0.05 M lysine-2% paraformaldehyde (w/v) (PLP) solution (15). Samples were washed overnight in phosphate-buffered saline with several changes and then incubated in a mixture of 15% gelatin (w/v)-10% glycerol (w/v) at 37 C for 24 h. After the samples embedded in gelatin were frozen in dry-ice acetone mixture, they were sectioned to 8- to 10 μm thickness with a Cryostat (Ames, Iowa). The sections were air-dried on microscope slide glasses which had been coated evenly with a thin film of 2% gelatin (w/v). They were then fixed again for 10 min with either (*a*) 2% paraformaldehyde or (*b*) PLP solution. After washing three times (15 min each) in phosphate-buffered saline solution, sections were incubated in fetal calf serum (10-fold dilution) and 0.05 M lysine solution at room temperature for 30 min in a moist chamber to avoid nonspecific binding between tissue and antiserum. After washing three times (15 min each) in phosphate-buffered saline, sections were incubated with the purified rabbit anti-β-amylase IgG (0.1–0.2 mg/ml) at room temperature for 40 min in a moist chamber, washed three times (15 min each) with phosphate-buffered saline, and then incubated with FITC-conjugated goat antirabbit IgG fraction (F/P = 1.6) (0.1 mg/ml) for 40 min. FITC-conjugated goat antirabbit IgG (Miles, Elkhart, Ind.) had been preliminarily fractionated by the acetone liver powder of rat and mouse to avoid the nonspecific binding to tissue. The sections were washed three more times (15 min each) in phosphate-buffered saline, and cover glasses were mounted with 90% glycerol (w/v). The following staining controls were routinely employed: (*a*) phosphate-buffered saline only; (*b*) FITC-conjugated goat antirabbit IgG only; and (*c*) nonimmune rabbit serum plus FITC-conjugated goat antirabbit IgG. Sections were then examined with a Tiyoda fluorescence microscope with oil immersion objective, and photographs taken in 2-min exposures (Kodak Ektachrome 200).

RESULTS

The specific cross-reactivity of the γ-globulin fraction of the rabbit anti-β-amylase serum was tested first. The Ouchterlony double immunodiffusion agar plate presented in Figure 1, A-1 clearly shows that the antiserum reacts only with β-amylase and that no precipitin line formed with the purified preparation of the rice seed α-amylase (*cf.* Fig. 1 of ref. 19). Reaction of the crude seed extracts with the antiserum resulted in the formation of an additional precipitin line on the immunodiffusion agar plate (Fig. 1, A-2). This line indicates the possible presence of extraneous antigenic principle(s) in the β-amylase preparation, which likely caused the development of an extra antibody. To purify the antiserum further, affinity column chromatography was employed (Fig. 2); extraneous antisera against the non-β-amylase fraction were effectively removed by this step, and the purified anti-β-amylase IgG (shadowed) was pooled and concentrated. In the Ouchterlony immunodiffusion agar plate precipitin lines formed between the IgG and either β-amylase or crude seed extracts are completely fused (Fig. 1, A-3). Also, immunoelectrophoresis experiments gave a single arc with each β-amylase and crude extract (Fig. 1B). The nonimmune rabbit serum does not form precipitin with any of the antigens, including a pure β-amylase preparation (not shown). It is evident that the antibody (IgG) obtained is monospecific toward β-amylase from rice seeds and, subsequently, we used it for the immunofluorescence labeling experiments to localize the enzyme in rice seeds.

Microphotographs (Fig. 3) are the results of the immunofluorescence labeling of thin sectioned rice seed specimens (1-day germination stage) treated with anti-β-amylase IgG followed by conjugation with the FITC-labeled goat antirabbit IgG. The freeze-sectioned seed specimens treated with either only FITC-antirabbit IgG (A, × 200), or nonimmune rabbit serum plus FITC-antirabbit IgG (C, × 400), show a strong autofluorescence of the cell walls; identical pictures were obtained by phosphate-buffered saline only (not shown). On the other hand, as clearly seen in the photographs (B, D, E, F), treatment of the thin sectioned seed specimens with anti-β-amylase IgG gave a prominent fluorescence labeling of the periphery of the starch granules in the entire

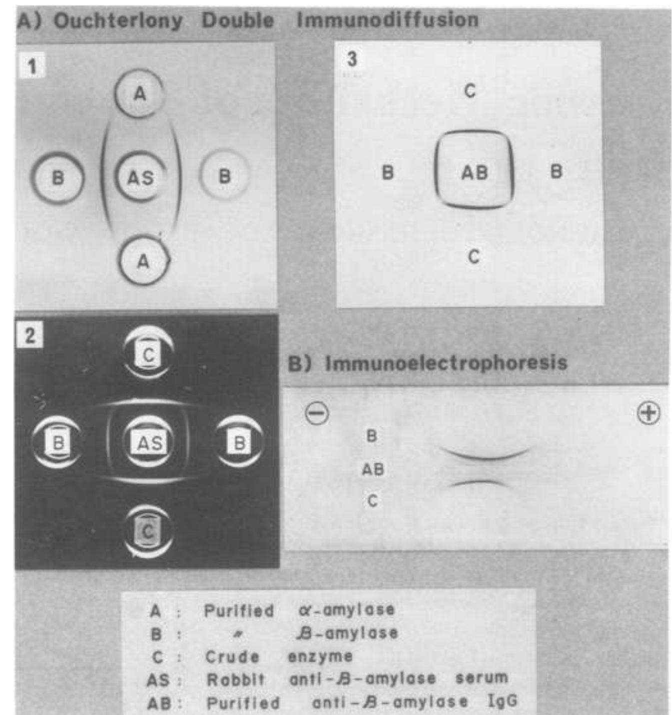

FIG. 1. Ouchterlony double immunodiffusion (A) and immunoelectrophoresis (B). A: purified α-amylase; B: purified β-amylase; C: crude extracts from germinating rice seed endosperm (7-day); AS: rabbit anti-β-amylase serum; AB: purified rabbit anti-β-amylase IgG. For other experimental details see text.

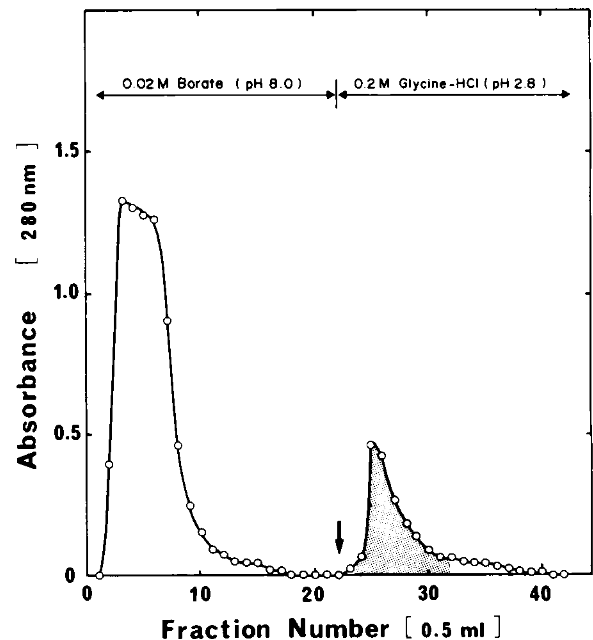

FIG. 2. Purification of rabbit anti-β-amylase IgG by affinity column chromatography. γ-Globulin fraction of rabbit anti-β-amylase serum was loaded onto a Sepharose 4B column to which a purified preparation of β-amylase was covalently bound. At arrow, eluting buffer of 0.2 M glycine-HCl (pH 2.8) was applied to disrupt the antigen-antibody complex.

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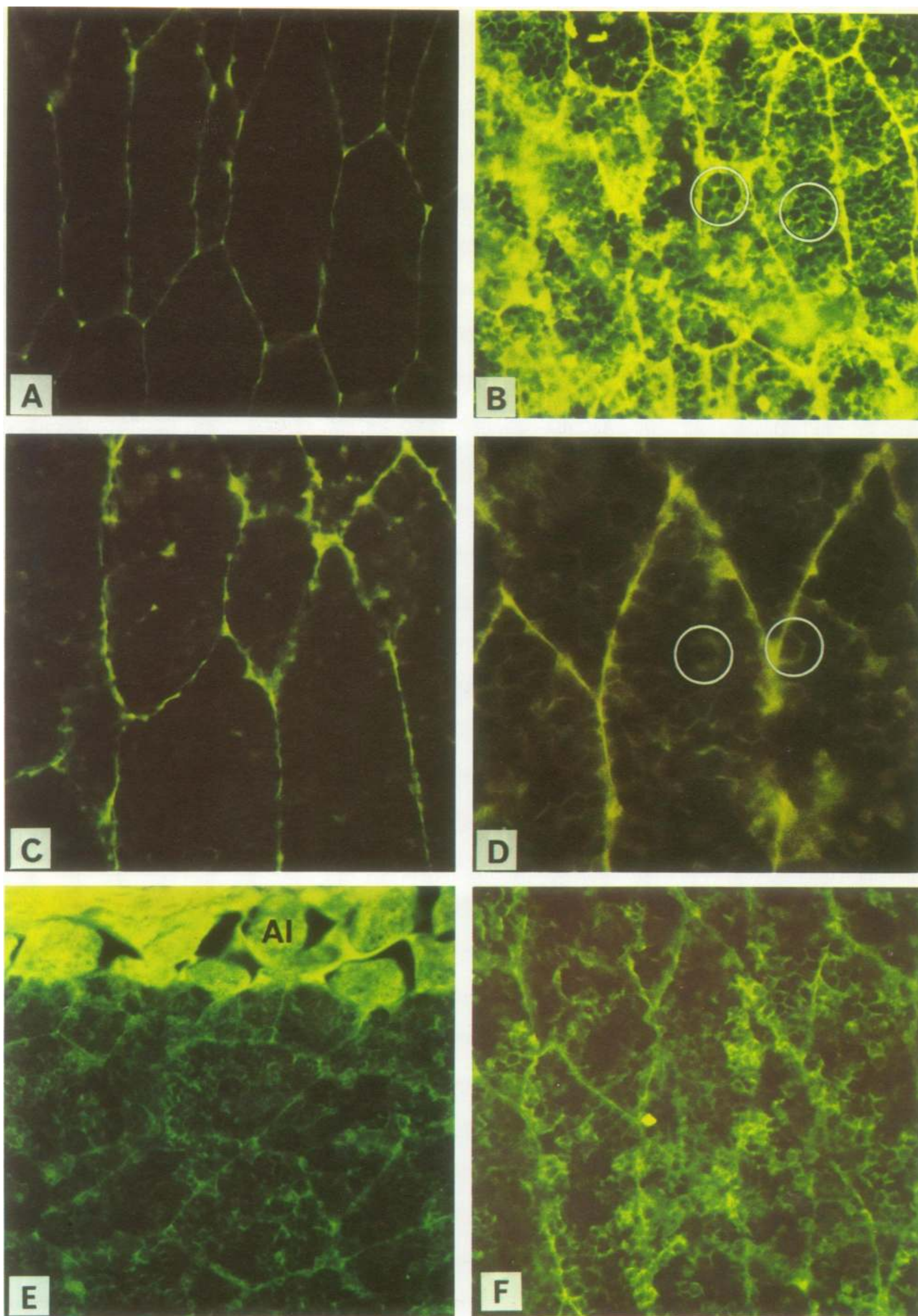

FIG. 3. Immunofluorescence localization of β-amylase in thin sections of rice seeds (1-day). Experimental details for the indirect immunofluorescent labeling of the thin sectioned seed specimens and subsequent microscope examinations are described in the text. Fixation procedures used were: A-D, PLP solution (10 min) and E and F, 2% paraformaldehyde (10 min), respectively. A: treatment by FITC-conjugated goat antirabbit IgG only; C: control serum (nonimmune rabbit serum) plus FITC-conjugated goat antirabbit IgG; B, D, E and F: treatment by rabbit anti-β-amylase IgG plus FITC-conjugated goat antirabbit IgG. B: amyloplast containing compounds starch is marked by circle. D: single starch grain is marked by circle. E: aleurone cell is marked by Al. Magnification: A, B, E, F (× 200); C and D (× 400).

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starchy cells. Not only single starch grains, but also the periphery of the amyloplasts containing compound starch grains in the starch cells, fluoresced (see circles in B and D). The aleurone layer in which aleurone grains are abundant is strongly fluorescent (Fig. 3E). Since essentially the same labeling picture can be obtained by treating the sections with the nonimmune rabbit serum (not shown), the aleurone cells are likely to be autofluorescent. The over-all results indicate therefore that β-amylase is specifically associated with starch granules in the starchy endosperm cells.

DISCUSSION

The classical investigations by Geddes (10) and Myrbäck and Neumüller (17) indicated that in cereal seeds β-amylase exists in an inactive latent form in association with proteins. Rowsell and Goad (23, 24) found that in wheat seeds the latent form of β-amylase bound with the protein (glutenin) molecules through disulfide linkages can be split by either reducing agents or a proteolytic enzyme such as papain to liberate active enzyme molecules. They concluded that the association of the enzyme with protein cannot be ascribed to the adherence of the protein component to starch granules. A later immunochemical study by Tronier and Ory (26) showed that in barley seeds, β-amylase associated with protein bodies can be released by treatment with β-mercaptoethanol. Although thorough investigations of the activation process of the latent form of β-amylase bound to protein bodies in germinating cereal seed tissue remain to be made, it is frequently emphasized that the presence of inactive β-amylase *in situ* is in a sharp contrast to that of α-amylase, which is newly synthesized during germination. The occurrence of protein bodies in starchy endosperm cells and their deposition during developmental stage of rice seeds are well recognized (16). Inasmuch as it has been shown that the β-amylase activity develops during the rice seed ripening process (2), it appears pertinent to localize β-amylase molecules in the starchy endosperm cells. Specific localization of α-amylase in aleurone cells has been reported by several investigators using immunohistochemical technique (12, 13). Our knowledge concerning the cellular localization of β-amylase in the endosperm cells is scanty, except for classical microchemical investigations carried out by Linderstrøm-Lang and associates (8, 14).

Development of the specific rabbit antiserum against the purified β-amylase from rice seeds has been of particular advantage for our experimental objectives; it is now clearly demonstrated that β-amylase is associated with starch granules uniformly present in the endosperm cells. It is likely that the immunochemically identifiable β-amylase is the inactive form of the enzyme, as we cannot detect the amylase activity in the starchy endosperm of 1-day stage rice seed (*cf.* Fig. 1 of ref. 20). This proposition can be further reinforced by an experiment of Daussant and Corrazier (6), showing that the inactive β-amylase isolated from the wheat seeds cross-reacts with the antiserum directed to the active enzyme molecules.

As the aleurone cells are strongly autofluorescent (Fig. 3E), we have to reserve our final conclusion whether *all* of the β-amylase is bound to starch granules. It is quite unlikely that the starch-bound β-amylase is derived from the enzyme molecules originally present in aleurone layers. From the structural dimension of protein bodies (16), it is also unlikely that the observed pictures are ascribed to the enzyme molecules bound to protein bodies adhering to the periphery of starch granules. On the other hand, it has been reported that cereal starch granules are coated with protein molecules and that amyloplasts have a membranous structural make-up (3, 4, 11). It is conceivable that the inactive β-amylase molecules constitute a part of such proteins surrounding the starch granules, and the elucidation of the nature of the binding of β-amylase with starch granules in particular relation to the mechanism of starch digestion is needed. One of the likely interpretations is that at the time of seed maturation the accumulation of starch granules is accompanied by the associated deposition of β-amylase on the periphery of starch grains. In this context, it may be recalled that starch synthase actively engaged in the amylose formation during the seed maturation remains tightly attached to the starch granules in the ripened dry cereal seeds (1). We postulate that at the onset of germination the activation and subsequent release of β-amylase from starch granules is the primary step of starch digestion. Since our previous observation has shown that in the early germinating stage the amylase activity originating primarily from the epithelium is spread to the entire region of endosperm (20), it can be suggested that the inactive β-amylase associated with starch granules in the endosperm cells is activated by agent(s), secreted from the epithelium, and eventually released. We also found that β-amylase activity is detectable in the scutellum including the epithelium (20). An important question that remains to be answered is whether or not there exists *de novo* biosynthesis of β-amylase in the scutellum of germinating rice seeds.

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