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Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds[1]

15. IMMUNOCHEMICAL STUDY ON MULTIPLE FORMS OF AMYLASE

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ABSTRACT

The formation of multiple forms of amylases in germinating rice (*Oryza sativa* L. cv Kimmaze) grains was examined by means of isoelectric focusing, cross-immunoelectrophoresis, and rocket-line immunoelectrophoresis followed by a reaction of enzymic characterization by using β-limit dextrin or starch as substrate. The constituents detected by isoelectric focusing were identified as three electrophoretically heterogeneous antigens. The major α-amylase bands A and B corresponded to a same antigen, the main portion of which was produced within 2 days' germination. The bulk of α-amylase D appeared between 2 and 4 days' germination. Component E, a debranching enzyme according to its action on the β-limit dextrin, already exists in the ungerminated seeds; its amount decreases within the first 2 days of germination and increases again thereafter.

Evidence showing that β-amylase (band C) is produced by the scutellum at an early stage of germination was provided. The enzyme appeared in a suspension of the scutellum after a prolonged incubation.

During the initial stage of rice grain germination, the scutellar epithelium produces multiple forms of α-amylase (12). It also produces β-amylase (14) although an inactive form of the enzyme exists already in the starchy endosperm (13). Electron microscopic investigations have shown that there is an active synthesis of hydrolytic enzymes, *i.e.* α-amylase in the scutellar epithelium at the onset of germination (1, 15). The *in vitro* protein synthesis directed by poly(A)-mRNA isolated from the scutellar tissues as well as the enzyme biosynthesis using the freshly dissected scutellar produced multiple forms of α-amylase (10, 11). Results of all these studies indicate that multiple genes appear to exist in the epithelium tissues directing the biosynthesis of isozymic α-amylase molecules. The elucidation of possible mechanism(s) controlling the gene expression in the germinating rice grain cells, in particular the differential synthesis of the multiple forms of α-amylase in the epithelium, remains an important problem to be solved. This communication deals with an experimental approach employing immunochemical techniques to characterize multiple forms of amylases (α and β) in whole rice grain extracts as well as in excised scutellar tissues (3, 9). Information from studies on the quantitation of multiple enzyme forms at different stages of grain germination will be of use for future analytical studies of the cell-free synthesis of amylase molecules. The study concerns also the characterization of the debranching enzyme (R enzyme) (6).

MATERIALS AND METHODS

Plant Growth and Preparation of Seed Extracts. Rice seeds (*Oryza sativa* L. cv Kimmaze) were soaked in 1% NaOCl solution for 15 min and, after thorough washing in tap water, the seeds were germinated on filter paper placed on sterilized cotton moistened with water in a Petri dish in a dark chamber at 30°C. Seeds were then harvested at the appropriate growing stages as indicated and frozen at −20°C. After removing shoots and roots, seeds (1 g fresh weight) were ground in a mortar using 1 ml of 0.05 M Tris-HCl (pH 5.7) buffer containing 3 mM $CaCl_2$ and 1 mM DTT at 0°C. The whole homogenates were centrifuged at 4°C (60,000*g*, 30 min) in order to obtain a clear supernatant solution.

Isoelectric Focusing on Polyacrylamide Gel. To detect multiple forms of amylase activity, the crude extracts of whole seed were applied to polyacrylamide gel isoelectric focusing (pH 4–6) essentially following the method reported previously (2). At the end of electrophoresis, gel samples were subjected to either zymogram staining or to cross-immunoelectrofocusing (see below). For making amylase zymograms, either soluble starch or β-limit dextrin coated on a glass plate was used as substrates, followed by the I_2-KI staining after a suitable incubation period.

Immunochemical Method. A homogeneous preparation of α-amylase (band A), as evidenced by the polyacrylamide gel isoelectric focusing (pH 4–6), was used for the immunization of rabbits to prepare the antiserum (11). The whole IS[2] was mostly used in the present investigation, but whenever necessary, IgG was purified from IS by affinity chromatography. The preparative method of IgG specific to the purified preparation of β-amylase was described previously (14).

Immunoabsorption combined with isoelectric focusing was carried out as described earlier except that the anti-rice α-amylase IS was used throughout this investigation. For characterizing β-amylase in the scutellar tissues, 50 freshly dissected scutellar tissues free from endosperm at the 4-d stage were carefully washed with 2 mM acetate buffer (pH 5.3) and suspended in 1 ml of the same solution. The bathing solution was replaced 5 times using 5 ml each of the buffer solution at 20, 40, 80, and 160 min of incubation time. The final bathing solution was treated with 1 mM EDTA

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[2] Abbreviations: IS, immune serum; IgG, immunoglobulin G.

solution overnight to inactivate partially the α-amylase activities, and an aliquot was applied to the polyacrylamide gel electrophoresis. This procedure is basically the same as that reported previously (9). Separate EDTA-treated extracts were incubated with the rabbit anti-β-amylase serum (IgG). At the end of electrophoretic runs, gels were used to prepare amylase zymograms.

Cross-immunoelectrofocusing was carried out according to Söderholm *et al.* (16). The technique combines the polyacrylamide gel isoelectric focusing with a second electrophoresis performed in a perpendicular direction to the first electrophoresis in an agarose gel containing IS. After isoelectric focusing, a 1-cm broad polyacrylamide gel strip was cut and deposited on the agarose gel containing IS before the second electrophoresis. In order to get optimal conditions for identifying the different antigens, various amounts of the seed extracts were applied to the first dimensional isoelectric focusing and different concentrations of IS were used in the second dimensional immunoelectrophoresis as indicated in each figure. Voltage and running time of electrophoresis varied as indicated in each experiment. At the end of immunoelectrophoresis, gel plates were subjected to the amylase characterization by soaking in a β-limit dextrin solution, followed by staining with an I_2-KI solution. Some of these plates were dried and stained with Coomassie brilliant blue.

Rocket-line immunoelectrophoresis was carried out according to Krøll (5). To characterize the different antigenic enzyme molecules, various concentrations of IS in the gel and of antigen in the strip gel were used as indicated in each figure. The wells were filled with the undiluted seed extracts or after their suitable dilution as indicated in the figure. Electrophoresis was carried out at 4°C for 14 h at 5 v/cm. Following electrophoresis, the gel plates were submitted to either protein staining with Coomassie brilliant blue or amylase characterization by soaking the gels in β-limit dextrin solution as substrate, followed by staining with an I_2-KI solution.

Enzymes. The pullulanase (*Aerobacter aerogenes*) was purchased from Sigma (No. 2138).

RESULTS AND DISCUSSION

α-Amylase and R Enzyme. The amylase zymogram patterns were examined after separation (pH 4–6) by isoelectric focusing of the proteins extracted from the whole grains taken at the 4-d germination stage. The basic isozyme pattern of the amylase activities using the soluble starch as substrate (Fig. 1a) is similar to our previous observations (*cf.* Fig. 2 of Ref. 12); three major bands (A, B, and C) and several minor bands were detectable. One of the additional major components was newly designated as D. By the immunoabsorption of the whole extract with the rabbit anti-α-amylase IS, all the components, except band C, disappeared (Fig. 1b).

Alternatively, by using the β-limit dextrin as substrate, the zymogram pattern was similar to Figure 1a, but band C was much less prominent; the faint band which is visible in its close vicinity is probably due to a minor α-amylase constituent. Furthermore, it was noted that some minor bands, designated as E, having specific blue coloration on the β-limit dextrin zymogram, appear (dotted arrows in Fig. 1c). This indicates that the bands may be due to the action of the debranching enzyme (R enzyme), splitting the α-(1–6)-D-glucosidic bonds in the β-limit dextrin molecules

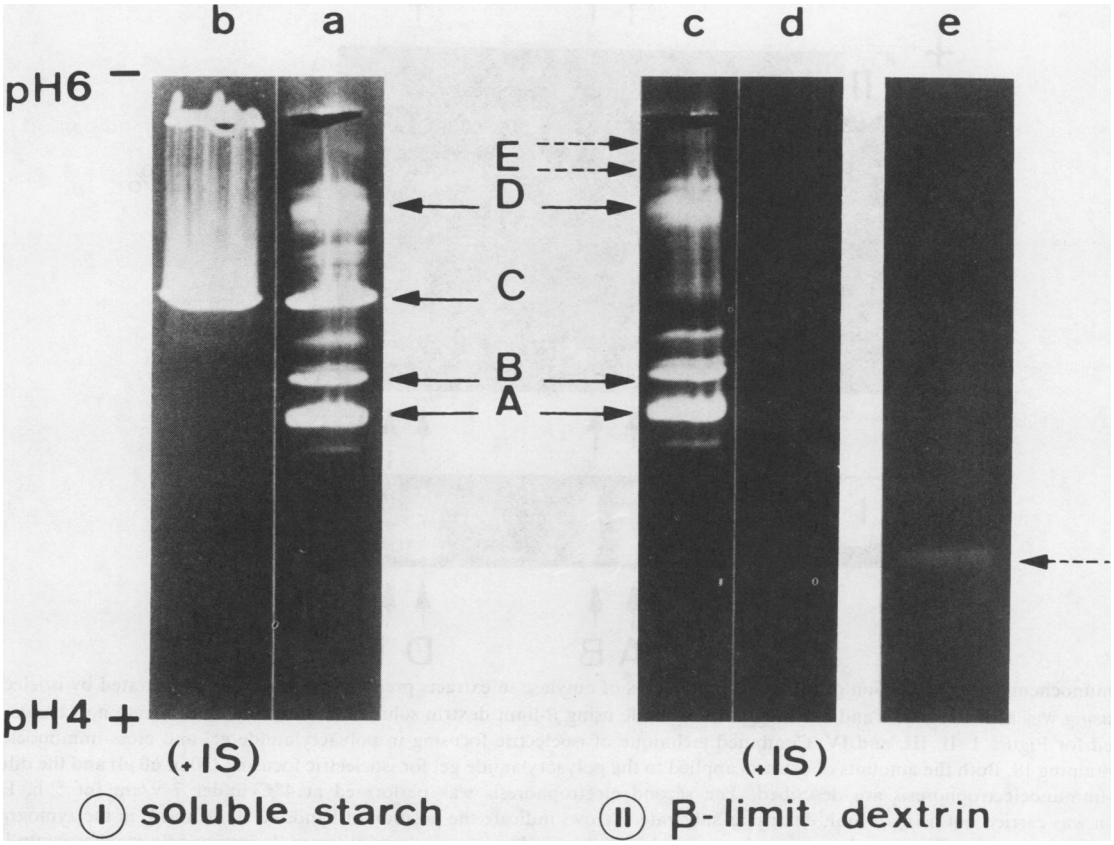

FIG. 1. Amylase zymogram of whole rice seed extracts (4 d) separated by polyacrylamide gel isoelectric focusing. a and c, Isoelectric focusing combined with immunoabsorption using nonimmune control rabbit serum. b and d, Isoelectric focusing combined with immunoabsorption using anti-rice α-amylase serum (IS). e, Isoelectric focusing of purified preparation of pullulanase. I, Gels were incubated with a soluble starch-coated glass plate. II, Gels were incubated with a β-limit dextrin-coated glass plate. Dotted arrows indicate bands with blue coloration. Other experimental details are described in the text.

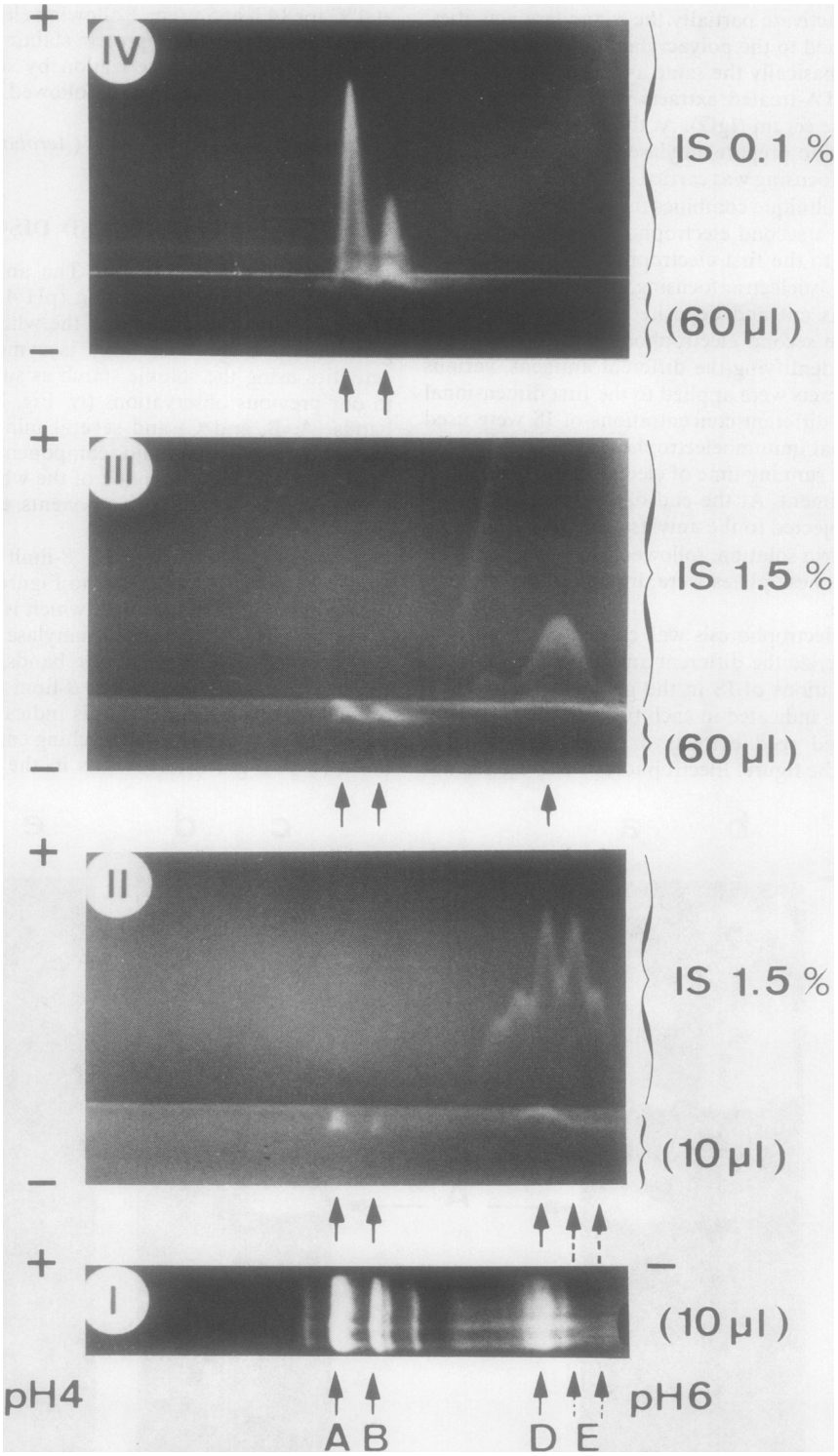

FIG. 2. Immunochemical identification of multiple components of amylase in extracts prepared from 4-d seeds separated by isoelectric focusing. I, Isoelectric focusing was as for Figure 1 and a zymogram was made using β-limit dextrin solution as substrate. Identification of bands A, B, D, and E are as described for Figure 1. II, III, and IV, Combined technique of isoelectric focusing in polyacrylamide gel and cross-immunoelectrophoresis in agarose gel containing IS. Both the amounts of extracts applied to the polyacrylamide gel for isoelectric focusing (10 or 60 µl) and the dilution of IS used in each cross-immunoelectrophoresis are described. The second electrophoresis was performed at 4°C under 7 v/cm for 5 h. Enzyme activity characterization was carried out using β-limit dextrin as substrate. Arrows indicate the position of bands A, B, D, and E in the zymogram as well as in the immunoelectropherograms. The dotted arrows indicate bands with blue coloration on the zymogram. In immunoelectropherogram II, the precipitin band with five more or less sharp peaks is bluish.

and allowing a greater incorporation of iodine. For testing this possibility, pullulanase from *A. aerogenes* was applied to isoelectric focusing; pullulanase is a debranching enzyme which hydrolyzes α-(1–6)-glucosidic bonds of pullulan as well as the β-limit dextrin molecules (8). A similar blue coloration was observed with the pullulanase preparation (dotted arrow in Fig. 1e). However, after

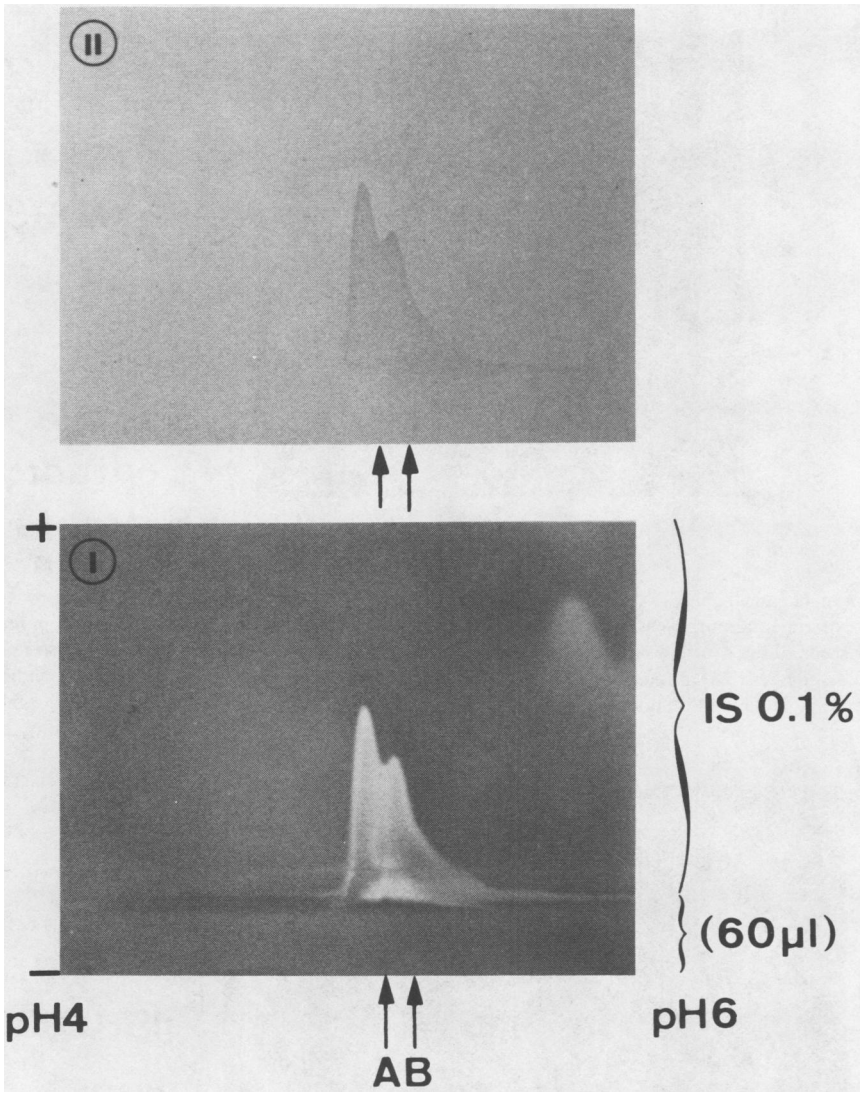

FIG. 3. Immunochemical comparison between α-amylase isozymes (A and B). Cross-immunoelectrophoresis was performed under the identical conditions as given for Figure 2IV; 60 μl of the crude extract of 4-d germinated rice seeds was applied to the polyacrylamide gel-isoelectric focusing separation and the agar gel containing 0.1% IS was employed for the second electrophoretic separation. However, the second electrophoresis was carried out at a lower voltage (2 v/cm) for 15 h. The plate was then stained for the enzyme activity characterization using β-limit dextrin as substrate (I), followed by the subsequent protein staining using Coomassie brilliant blue (II).

immunoabsorption of the whole extract with the IS, none of the bands was detectable on the β-limit dextrin zymogram (Fig. 1d). These results indicate that, consistent with our previous observation (12), bands A and B and some additional components are α-amylase, while band C is β-amylase. In addition, some minor bands, recognizable by the use of β-limit dextrin gel plate, were tentatively characterized as debranching enzyme (R enzyme). Results further suggest that several α-amylase constituents are antigenically related to each other and that the IS responds to various antigenic species bearing activities of α-amylase as well as debranching enzyme.

For characterizing multiple antigenic components and studying their relationships, cross-immunoelectrofocusing was performed (Fig. 2). This technique proved to be particularly powerful for characterizing the amylase isozyme components in cereal seeds during developmental and germination stages (2, 3, 7). In the present experiment, 60 μl (Experiments III and IV) and 10 μl (Experiment II) each of the whole seed extracts were first subjected to isoelectric focusing (pH 4–6) and the concentration of IS used for the subsequent cross-immunoelectrophoresis ranged from 0.1% to 1.5%. Two precipitin bands with α-amylase activities were detected in Experiment IV. Evidently, they correspond to bands A and B. By increasing the concentration of IS to 1.5%, a third precipitin band bearing the α-amylase activities appears (Experiment III). The position of the precipitin band in the immunoelectropherogram corresponds to band D and other minor constituents, as can be seen from a shoulder on the left side band. In Experiment III, a fourth precipitin band bearing the amylase activity was visible. However, under the experimental conditions employed, the amount of this antigen was too high for its precise characterization. By decreasing the amount of the extracts in relation to IS (1.5% IS *versus* 10 μl extract), the precipitin band was clearly visible (Experiment II). The blue coloration of the precipitin band appears to indicate that the antigen present in the precipitated immunocomplex is debranching enzyme (R enzyme). Inasmuch as the precipitin band forms a continuous line containing more or less five sharp peaks, it is conceivable that the debranching enzyme contains several molecular forms, differing in their isoelectric point. However, some of these forms cannot be detected on the zymogram (*cf.* Figs. 2I and 1c), probably because

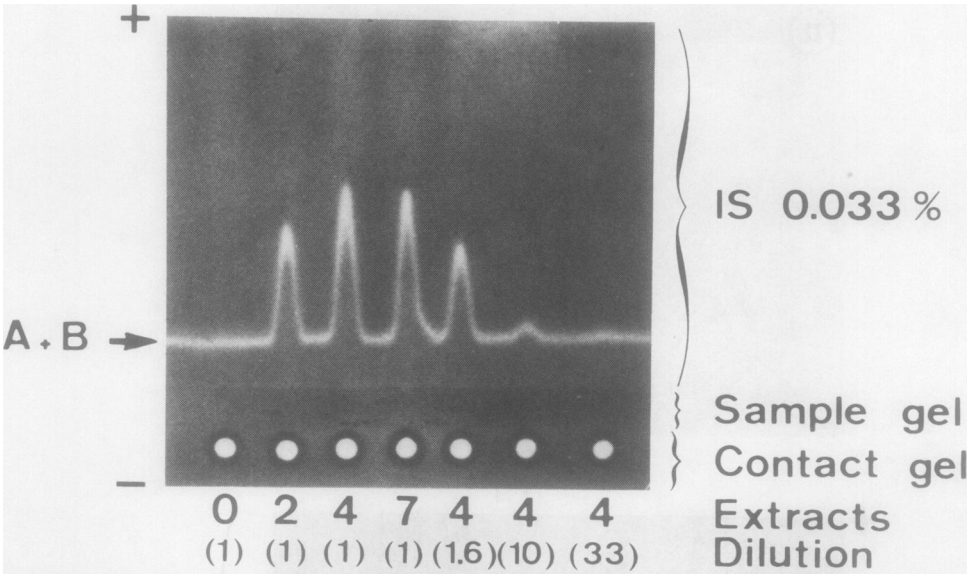

FIG. 4. Quantitative immunochemical analysis of α-amylase isozymes (A and B) during various germination stages. Rocket-line immunoelectrophoresis was employed for the experimental purpose. Agarose gel containing 0.033% IS was used in the second electrophoresis. Sample gel contained the extracts of 4-d germinated seeds, diluted 6-fold in the agarose gel kept at 50°C. Wells cut in the contact gel were filled up with the extracts of ungerminated (○) or germinated seeds of various stage (2-, 4-, and 7-d), which were diluted appropriately as indicated (1.6-, 10-, and 33-fold). Electrophoresis was carried out at 4°C for 14 h under 5 v/cm. Enzyme activity characterization was carried out on the gel after electrophoresis by using β-limit dextrin as substrate.

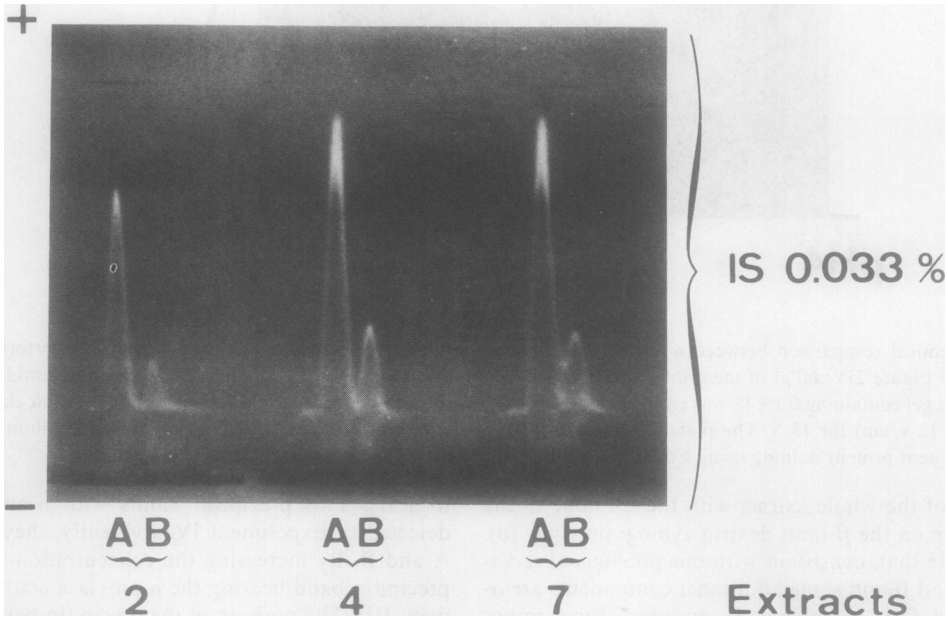

FIG. 5. Quantitative immunochemical comparison between α-amylase isozymes (A and B) in seed extracts during various germination stages. The cross-immunoelectrofocusing technique was performed for the experimental purpose. Experimental procedures of the isoelectric focusing were the same as shown in Figure 1, using the extracts prepared from 2-, 4-, and 7-d germinated seeds. The cross-immunoelectrophoresis was carried out in an agarose gel containing 0.033% IS. A part of the polyacrylamide gels containing A and B was cut after isoelectric focusing and deposited upside down on the agarose gel. Electrophoresis was carried out at 4°C for 14 h under 6 v/cm. After the electrophoresis, the enzyme activity characterization was performed using β-limit dextrin as substrate.

they are masked by α-amylase molecules having a similar isoelectric point. Hereafter, this precipitin band is designated as E.

Throughout the germinating stages tested, the isozyme components A and B clearly are most prominent among total α-amylase constituents identifiable by isoelectric focusing, and we attempted to characterize them more precisely. To compare the antigenicities of bands A and B, the cross-immunoelectrofocusing was carried out under conditions slightly different from those shown in Figure 2IV (lower voltage and longer electrophoresis running time) (Fig. 3). Both the characterization of enzyme activities (I) and the protein staining (II) were carried out on the same plate, and the identical patterns observed show that the Coomassie brilliant blue-stainable two protein molecules bear the α-amylase activities (A and B). The complete fusion of the precipitin line between A and B indicates that there is no antigenic difference between the two molecular species.

In an attempt to compare the whole amounts of A and B at different germination stages, the rocket line-immunoelectropho-

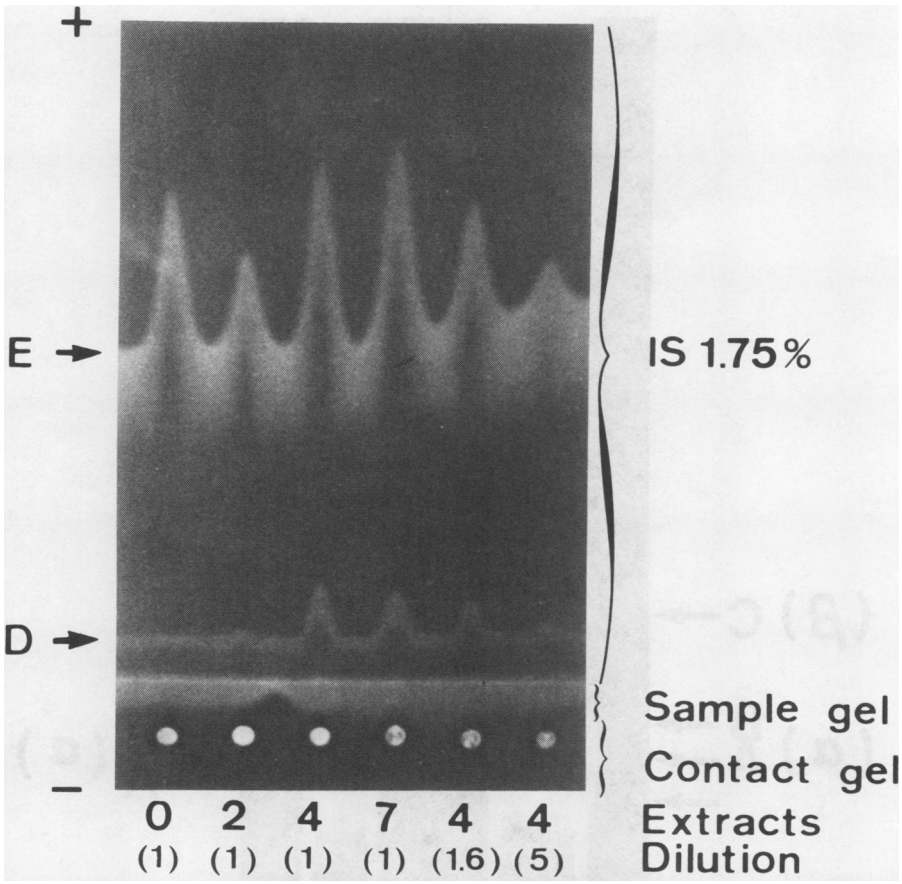

FIG. 6. Quantitative immunochemical analysis of antigenic components (D and E) during various germination stages. Basic experimental procedures of rocket-line immunoelectrophoresis were the same as given in Figure 4. The agarose gel containing 1.75% IS was used. The sample gel contained the extract of 4-d germinated seeds, diluted 4-fold in the agarose gel and kept at 50°C. Wells cut in the contact gel were filled up with the extracts of ungerminated seeds (O) or germinated seeds of various stages (2-, 4-, and 7-d), which were diluted appropriately as indicated (1.6- and 5-fold). Electrophoresis was carried out at 4°C for 14 h under 5 v/cm. Enzyme activity characterization was carried out on the gel after electrophoresis by using β-limit dextrin as substrate.

resis was used, A and B being determined as a single antigen (Fig. 4). On the other hand, amounts of A and B were separately assayed and compared with each other by using cross-immunoelectrophoresis (Fig. 5). For detecting the enzyme activities, the agar plates were stained using β-limit dextrin as substrate. Antigens A and B are totally absent in the ungerminated dry seed extracts, and their content increased markedly within the first 2 d of germination, and to a much lesser extent afterwards (Fig. 4). During the seedling growth of 2 to 7 d, the amount of isozyme A is much greater than that of B, while the level of both components reached the maximum at about 4 d (Fig. 5). These results are essentially consistent with our previous observation (Fig. 2 of Ref. 12).

The developmental formation of the additional components which act on β-limit dextrin (D and E) was studied by using the rocket-line immunoelectrophoresis under the experimental conditions used in Figure 6. In the experiment, D and E were characterized on the same plate. Results show that component D is produced between 2 and 4 d of the germination stage, its formation being later than that of A and B. The temporal difference in the production of these two classes of amylase may reflect the tissue-specific synthesis as well as the localization of the enzyme; our previous experiment has shown that, although the initial site of the α-amylase formation in the germinating rice seed is in the scutellar epithelium, in the later stage (after 4 d) some additional enzyme species appear to be synthesized, presumably in the aleurone layers (12). In the present investigation employing line immunoelectrophoresis, isozymes A, B, and E, but not D, were found in the extracts of the freshly dissected "scutella" at the 4-d stage (data not shown). The absence of component D in the scutellum can be seen from the result in Figure 7 (see below). Therefore, if D only is synthesized in the aleurone, it is not surprising that it is only detectable in the zymogram after the appearance of A and B. We have shown that aleurone layers produce α-amylase several days after the enzyme production initiated in the scutellum (12).

In contrast to the isozymic components A, B, and D, antigen E (R enzyme) was found to exist in the ungerminated seeds, its amount decreasing during the first day of germination and increasing thereafter (*cf.* Fig. 6). R enzyme was reported to exist in the ripened dry rice grain in both active and inactive forms, the latter being extractable by means of the reducing agents or papain; during the grain germination, an increase of the R enzyme activities can be ascribed to a conversion of the inactive form into the active form (17, 18).

β-Amylase. Our previous experiments have shown that band C (β-amylase) is detectable in extracts from both scutellum and endosperm tissues (9). Therefore, it is likely that the enzyme in the latter tissue is derived from the latent form associated with the starch granules after being activated by the proteolytic digestion. On the other hand, the enzymic band detectable in the scutellar extracts is presumably ascribed to the enzyme molecules newly synthesized *in situ* and secreted into the endosperm tissues (14). An important question arises as to whether or not these two forms

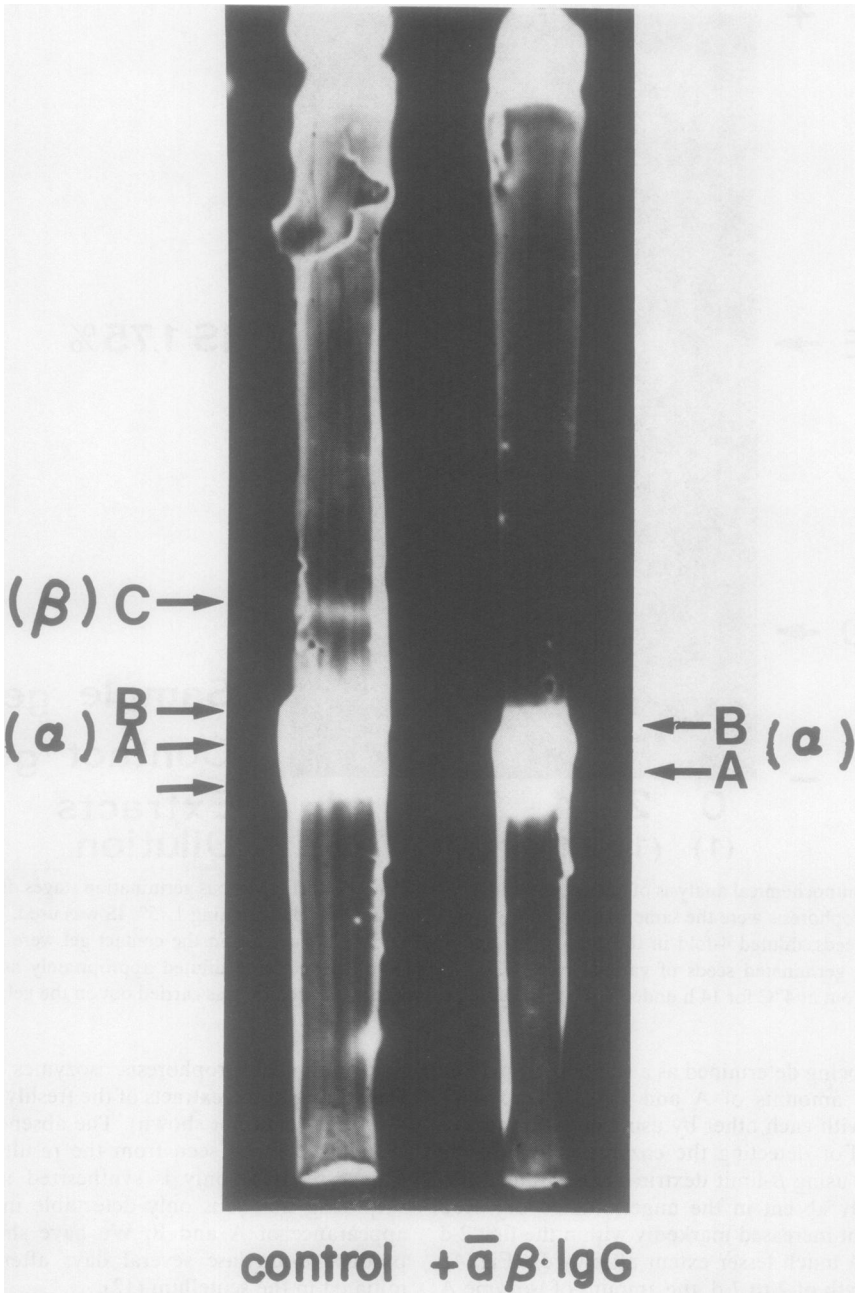

FIG. 7. Immunochemical identification for secretion of β-amylase from endosperm-free scutellum. Experimental details for bathing 50 pieces of freshly dissected rice seed scutella in 2 mM acetate buffer (pH 5.3) and time-dependent replacement of the bathing buffer solution are explained in text. The final bathing solution (160 min) was treated with 1 mM EDTA overnight and an aliquot was applied to the polyacrylamide gel electrophoresis, followed by making zymograms as shown in Figure 1. To demonstrate the presence of β-amylase in the medium, anti-β-amylase IgG was added to the bathing solution and applied to the gel electrophoresis.

of β-amylase are structurally identical. Freshly dissected scutellar segments were placed in a buffer solution; after replacing the bathing medium several times, the outside medium was subjected to the zymogram test. Upon a prolonged incubation for 160 min, β-amylase band (C) was visible in addition to the two major α-amylase bands, A and B, and a minor band beneath A, whereas β-amylase completely disappears by the treatment with anti-β-amylase IgG (Fig. 7). However, the release of β-amylase from the scutellum was much weaker compared to the α-amylase release (*cf.* 9). A question remains whether or not its synthesis is due to the mRNA remaining from the developing stage of the seed or to the newly formed mRNA. The direct answer to this can be provided from the studies on the cell-free synthesis of β-amylase directed by mRNA isolated from scutellum, which are now in progress.

Assessment. The overall experimental results obtained in the present study show that the multiple forms of the β-limit dextrin-degrading enzymes detectable in the germinating rice seeds contain at least three classes of antigenic molecules, each of them being electrophoretically distinguishable; during the germination step, their amounts change, which presumably reflects the pattern of enzyme synthesis and degradation. Two of these antigens are definitely α-amylase and the third component is likely to be debranching enzyme (R enzyme) as evidenced from its enzymic properties. In conjunction with our previous investigations, we concur that the site of their synthesis except for one antigen is in

the scutellar epithelium and the enzyme molecules will be eventually secreted into the endospermic tissues.

We have obtained preliminary experimental evidence indicating that the synthesis of each of two major components (A and B) is directed by distinct DNA (mRNA) (10), and further experiments are needed to examine the nature of the genetic message determining the formation of additional α-amylase species. On the other hand, the magnitude of the synthesis and secretion of β-amylase in the scutellar tissue does not seem to correspond to that occurring in the endosperm tissues, although our separate experiments have shown that β-amylase is also synthesized in ER like that of α-amylase (9). In this context, it is of interest to note that using the developing barley seed endosperm Johanssen *et al.* (4) reported the *in vitro* synthesis of β-amylase directed by the polysomes isolated from the ER fractions.

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