Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds

III. α-AMYLASE ISOBRETS

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ABSTRACT

The formation of amylase isozymes in germinating rice (Oryza sativa) seeds was studied by isoelectric focusing on polyacrylamide gel disc electrophoresis. Time sequence comparisons of the amylase zymogram were made between extracts from gibberellic acid-treated embryoless and embryo-attached half-endosperm of rice seeds. In both cases, 4 major and 9 to 10 minor isozyme bands were detectable at the maximal stage of the enzyme induction. However, in the embryo-attached half-seeds, bands started to diminish after the 5th day of incubation, in agreement with the results of time sequence analyses of enzyme activities. Nearly identical patterns of amylase isozyme bands on a polyacrylamide gel disc electrophoresis in combination with isoelectric focusing indicate the intrinsic role of gibberellic acid in the starch breakdown in germinating rice seeds. We tentatively assign the newly synthesized enzymes to be α-amylases based on experimental results concerning the stability of the preparation on a prolonged treatment at pH 3.3 and the stability on heat treatment for 15 minutes at 70°C.

Our previous experimental results on both chemical analyses and determination of enzyme activities have shown that the amylolytic pathway is the major mechanism for the breakdown of reserve starch in endosperm tissues of germinating rice seeds (17, 18). This finding is in good agreement with results reported by several workers on the formation of α-amylase in germinating cereal seeds, i.e., barley and rice, induced by gibberellic acid (2, 16, 19, 20, 22-24, 29-32, 35). A series of extensive studies carried out by Varner and his associates (3, 4, 6, 30-33) have well established the synthesis de novo of α-amylase in germinating barley seeds. The pattern of the enhancement of α-amylase activities in embryoless half-seeds in response to GA treatment was found to be analogous to that occurring in the intact germinating seeds. They have proposed that GA moves from embryo to aleurone cells, where it promotes the synthesis of α-amylase. The production of GA in embryos of developing barley seeds has been demonstrated by Radley (26, 27). However, there is no direct proof that the biochemical events occurring in intact seeds and that occurring in the GA-treated half-seeds are identical. This question must be critically examined, because isozymes of α-amylase were detected in the GA-treated embryoless half-seeds as well as in germinating intact seeds of barley (7, 14, 15, 21, 33). In an experiment reported in this paper, we have studied the time sequence change of amylase isozymes in GA-treated embryoless and in intact endosperm of rice seeds, to gain deeper insight into the enzymic mechanism of the hydrolytic breakdown of reserve starch. The isoelectric focusing technique (12) on a polyacrylamide gel disc electrophoresis proved to be advantageous for detection of many isozymes (13, 34).

MATERIALS AND METHODS

Half-endosperm Method of Germinating Rice Seed. Seeds of rice plants, Oryza sativa L. var. Fujiminori harvested in September 1969, were used throughout this study. Seeds were dehulled by hand, and the whole seeds were cut into two parts with a razor blade. Both embryoless and embryo-attached half-seeds were sterilized by soaking for 1 min in absolute ethanol and then dipping for 20 min in an aqueous solution of 10% (w/v) bleaching powder (NaOCl) (14). After thoroughly rinsing with sterilized distilled H2O, 10 each of embryoless and embryo-attached half-seeds were placed aseptically in 10-ml flasks containing 3 ml of either 2 × 10^-2 µg GA ml or distilled H2O, previously sterilized by autoclaving at 120°C for 15 min. Then, the contents of the flasks were aseptically incubated at 30°C. No discernible changes occurred in embryoless half-seeds, without the addition of GA. However, the dissolution of starch reserve occurred in the (+GA) system after 5 days of incubation. The development of both shoot and root in embryo-attached half-seeds was essentially the same as that occurring in normally germinating seeds.

Enzyme Assays. At each germination stage, the seed pieces were removed from the incubation medium and were ground in a chilled mortar with 3.0 ml of 0.01 M tris-HCl buffer (pH 8.5). After centrifugation of the homogenate at 15,000 rpm for 15 min, an aliquot of the supernatant solution was used for the assay of α-amylase activities by the method of Shuster and Gifford (28). An aliquot of each incubation medium was assayed for extracellular enzyme activity. As a quantitative measure of the relative enzyme activities, unit activities (mg of starch broken down per seed) were calculated from the data for 5 min of incubation at 30°C (cf. 17).

Measurements of enzyme activities were also carried out by determining the increase in the amount of reducing sugars formed by the method of Bernfeld (1) with maltose as reference. Absorbancy at 540 nm was measured by a Hitachi model 356 two wave length spectrophotometer.

In order to detect the possible presence of β-amylase in the crude extract, both heat and low pH treatments were carried out following the method of Paleg (23). To an aliquot (1.0 ml) of the crude extract (I) was added 1.0 ml of 0.01 M Ca-acetate, and the mixture incubated at 70°C for 15 min. Then the mixture was placed in an ice bath until used for measurements of enzyme activity.
activities (III). Another 1-ml portion of the crude extract was brought to pH 3.3 by addition of 0.1 N HCl and placed at 0 °C for 24 hr. Then, the pH was raised to 4.5 by adding 0.1 N NaOH, the preparation was diluted to 2 ml with water, and the diluted preparation was used for enzyme assays (II).

Isoelectric Focusing on Polyacrylamide Gel Disc Electrophoresis. Polyacrylamide disc electrophoresis was performed according to the method of Davis after some modifications (5). A solution containing 8% Ampholine (pH 5–8 or 4–6, LKB, Sweden), 0.004% riboflavin, 30% cyanogum-41, and H2O (1:1:2:4, v/v) was photopolymerized in glass tubes (5 × 80 mm). A spacer gel (10-15 mm) with 0.2 m ethylenediamine was added. Then, gel tubes were mounted to an electrophoresis apparatus, and a buffer solution of 0.2 m acetic acid containing 1 mM CaCl2 and 0.2 m ethylenediamine was added to the anode (lower) and cathode (upper) vessels. Electrophoresis was performed at the constant voltage of 200 V for 4 to 5 hr at 4°C. At the end of run, the gels were removed thoroughly rinsed with 1.0 M acetic buffer (pH 5.3) and stained for amylase as described below.

Amylase Zymogram. Banding patterns of amylase were detected by the technique developed by Momotani (personal communication). A glass plate (8 × 9 cm) with a thin film of 1% egg albumin as an adhesive was heated at 120°C for 1 hr. Then, a thin layer of a solution containing 30% cyanogum-41, 1.0 M acetic buffer (pH 5.3), 0.004% riboflavin, and 1.5% soluble starch (2.5:1:1:4, v/v) and a few drops of N,N,N',N'-tetramethyl ethylenediamine was added to each plate and photopolymerized. The gels to be tested for amylase were placed between two such starch-acrylamide plates in sandwich form and incubated for 30 min at 30°C. Then the plates were stained with the I2-HCl solution. Electrophoresed zones containing amylase

![Figure 1: Time sequence analysis of amylase activity of embryoless (with or without GA treatment) and embryo-attached (without GA treatment) half-endosperm tissue of rice seeds. Details of experimental procedures are described in the text. The enzyme activity is expressed as mg starch broken down per grain (30°C for 5 min), calculated from the achromatic colorimetry (absorbancy decrements at 620 nm).](image1)

![Figure 2: Effect of heat and low pH (3.3) treatment on amylase activities of crude extract from embryoless (with GA treatment) (A) and embryo-attached (without GA treatment) (B) half-endosperm tissue of rice seeds. Details of experimental procedures are described in the text. I: 5 μl of crude extract; II: 10 μl of heat-treated preparation (70°C for 15 min); III: 10 μl of low pH-treated preparation (pH 3.3, 0°C for 24 hr); Blank: no enzyme.](image2)
activities hydrolyzed the starch film and appeared as decolorized bands on a blue background.

RESULTS

Results presented in Figure 1 show the time sequence analyses of amylase activity of variously treated endosperm samples, employing the I₂-starch decoloration method. In both embryoless (+GA) and embryo-attached (–GA) half-seed endosperms, a marked increase in enzyme activity occurred after a lag period of about 1 day. However, there was a notable difference between the two systems. Amylase activity in the medium of GA-treated, embryoless endosperm tissue showed a nearly linear increase between 3 and 8 days, while that from the seed extract increased slightly between 3 and 6 days and declined thereafter. After 8 days essentially no starch-degrading activity was detectable in the absence of GA. In the embryo-attached endosperm, on the other hand, the enzyme activity began to level off after about 4 to 5 days; the pattern was similar to the one observed in intact germinating seeds (cf. Fig. 3 of Ref. 17). Practically no enzyme activity was measurable in the medium containing the embryo-attached endosperm tissue.

That the enzyme examined is an α-amylase is suggested by the results shown in Figure 2. The breakdown of soluble starch by the crude extract from both GA-treated embryoless and embryo-attached half-seeds as measured by the I₂-starch reaction (absorbancy at 620 nm) was hardly affected by heating at 70°C for 15 min. But lowering the pH of the preparation to 3.3 resulted in a drastic decline in enzyme activity. An identical trend was observed by analyzing the maltose formed (1). It is thus implied that β-amylase may play a minor role in hydrolyzing the reserve starch in germinating rice seed endosperm, unlike the case with barley seeds (23). By comparing the results of the two methods of detecting starch hydrolysis as presented in Figure 2, it will be noted that the amount of low molecular weight reducing sugars formed accounts for only a part of the total starch broken down in seeds.

Zymograms illustrated in Figures 3 and 4 are the results of time sequence analyses of α-amylase isozymes in both embryoless (+GA) and embryo-attached (–GA) half rice seeds by polyacrylamide gel isoelectric focusing. Conclusions that can be drawn are: (a) In both tissues no α-amylase bands are detectable in the dry seed extract (0-day) (pH 4–6) and the appearance of the enzyme activities (A and B) starts within a day. (b) There are 4 major prominent α-amylase bands, A, B, C, and D, and additional 9–10 weak bands after 3 to 5 days in both tissues. As revealed by their relative mobilities, these bands appear to correspond to each other in two systems, although there are some slight differences between the two. (c) In the embryo-attached endosperm extract, isozyme bands start to disappear after about 5 days, while in the GA-treated embryoless endosperm most bands remain unchanged during this period of time. These observations are in good agreement with the results of the time sequence analyses of enzyme activities (cf. Fig. 1).

DISCUSSION

The formation of α-amylase isozymes in germinating barley seeds was first noted by Frydenberg and Nielsen (7). Later studies by Momotani (14, 15) and Onckelen and Verbeek (21)
involved the use of GA-treated half-seeds. The latter workers have also described the presence of five isozymes in crude extracts from developing seeds. However, details on the correspondence of isozyme bands to those in the GA-treated half-seeds are missing. Based on technical problems inherent in the present experiments, it is difficult to establish that the α-amylase isozymes from embryoless (+GA) half-seeds are truly identical to those from embryo-attached half-seeds. In fact, it appears that there are some details which differ slightly, but we are inclined to speculate that the patterns of α-amylase isozymes from the two systems are basically similar, if not identical.

The inducible formation of α-amylase in barley half-seeds due to the exogenous addition of GA has been taken as circumstantial evidence for the promoting effect of GA on the enzyme synthesis in embryo. Two lines of experimental results appear to substantiate this thesis; (a) production of GA in the scutellum of barley embryos during the onset of germination (22, 26, 27), and (b) the ultrastructural studies on the aleurone cells of GA-treated barley half-seeds in comparison with that of normal seeds (9-11, 25). Therefore, while there remains much to be studied to correlate the biochemical events occurring in the germinating cereal seeds and the accompanying morphological changes, we consider that our present finding provides additional experimental evidence for the intrinsic role of GA in the process of the breakdown of reserve starch in germinating cereal grains.

The nature and the physiological function of multiple forms of α-amylase that occur in germinating cereal seeds cannot, at present, be assessed. Some of the isozymes detected might have resulted from the degradation with or without modification of the enzyme molecules during the preparative steps. Among some possibilities, the proteolytic breakdown of the newly synthesized α-amylase by a similarly GA-induced protease (8) might have caused the formation of multiple forms of the enzyme that are readily detectable by gel electrophoresis. As yet we presume that some of these isozymes are functioning intracellularly. Our recent experiments (29) have demonstrated that the α-amylase isozymes formed in the GA-treated barley endosperm are different in their action patterns as well as their kinetic properties. It is thus probable that various types of α-amylase are functioning cooperatively in the complete breakdown of reserve starch in the germinating rice seeds.

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LITERATURE CITED


