Enzymic Mechanisms of Starch Breakdown in Germinating Rice Seeds

7. AMYLASE FORMATION IN THE EPITHELIUM

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ABSTRACT

The time sequence analysis of the starch digestion pattern of the thin sectioned germinating rice (Oryza sativa L.) seed specimens using the starch film method showed that at the initial stage amylase activity was almost exclusively localized in the epithelium septum between the scutellum and endosperm. Starch breakdown in the endosperm tissues began afterward; amylase activity in the aleurone layers was detectable only after 2 days. Polyacrylamide gel electrophoresing (pH 4 to 6) revealed nearly the same zymogram patterns between endosperm and scutellum extracts, although additional amylase bands appeared in the endosperm extracts at later germination stages (4 to 6 days). These are presumably attributable to the newly synthesized enzyme molecules in the aleurone cells.

Germination of starchy cereal seeds is of classical interest in plant biological research. Despite extensive investigations in a variety of disciplines (e.g., anatomical, physiological, biochemical, and enzymic), the precise nature of starch breakdown is not entirely clear. Although there is a good possibility that each specific cereal seed has its characteristic machinery for hydrolyzing reserve materials, so far no unified mechanism has been presented in different kinds of cereal seeds relating to the enzymic hydrolysis of reserve starch.

Based on histological observations of germinating barley seeds, Brown and Morris (3) reported in 1890 that the site of synthesis of diastatic enzyme is in the epithelium of the scutellum; since then this unique feature of the epithelium has received much attention by several investigators (17, 23). In 1960, Dure (6) reported that in germinating maize seeds, the site of α-amylase (EC 3.2.1.1) biosynthesis was restricted to the scutellum, whereas β-amylase (EC 3.2.1.2) was localized in the endosperm. The crucial role of embryonic tissues (including the scutellum) in the digestion of starch of germinating cereal seeds has been proposed by several investigators. Briggs (2) reported that in malt, the α-amylase activity located in the embryo occupied about 7% of the total activity of the seed, and that 6.5% of the endospermic α-amylase was of embryonic origin. The scutellum is part of the embryo, and is clearly distinguishable from the embryonic axis, but analytical study of enzyme reactions occurring in epithelium is technically quite difficult because of the small size of the tissue.

Since the discovery of gibberellin, enhanced biosynthesis of α-amylase and other hydrolases in aleurone cells of barley seeds (4, 7), many investigations have focused at the molecular level on the intrinsic role of the hormone in the de novo synthesis of the enzyme (4, 8, 10, 12). There is ample experimental evidence showing the site of GA synthesis to be in the embryo; it is hypothesized that the hormone is transported to aleurone cells where hydrolytic enzymes including amylase are synthesized in situ (9, 14, 19, 20). The important role of aleurone layers in the enzymic starch digestion revealed by such studies appears to be consistent with the classical report of Linderstrøm-Lang and Engel (13), dealing with the ultramicroscopic examination of amylase localization in malt. The over-all picture is not necessarily consistent with the above described pattern of starch digestion initiating at the embryonic tissues, studied by previous workers. In work reported in this communication, we attempted to reexamine, employing a starch film technique, the initial site of amylase formation in germinating rice seeds.

MATERIALS AND METHODS

Growth of Plants and Preparation of Crude Enzyme. Rice seeds (Oryza sativa L. cv. Kimmazé) germinated in a dark chamber at 30 C were harvested at the appropriate stage (15), scutellum and endosperm tissues dissected from the seeds were used for analytical studies (16). It is relatively easy to separate the two tissues after the 2nd day of inhibition. To avoid contamination with endosperm, scutella were thoroughly washed with 50 mm Tris-HCl buffer (pH 7.0) containing 3 mm CaCl2 and 4 mm NaCl. One hundred each of isolated scutellar and endospermic tissues were then homogenized using 2 and 5 ml, respectively, of the buffer solution; the homogenates were centrifuged at 15,000g for 15 min. An aliquot of the supernatant fraction was passed through a column of Sephadex G-25 (1.0 × 9.0 cm) equilibrated with the above buffer solution. The eluate served as the crude enzyme preparation.

Enzyme Assays. α-Amylase activity was determined according to the method of Okamoto and Akazawa (18), using β-limit dextrin as substrate. β-limit dextrin was prepared by hydrolyzing potato starch solution (2% w/v) in 50 mm acetate buffer (pH 5.3), using crystalline potato β-amylase free from α-amylase (24 hr, 30 C). 0.3% β-limit dextrin dissolved in 50 mm acetate buffer (pH 5.3) containing 1 mm KH2PO4-2 mm CaCl2·2H2O served as the substrate. The reaction mixture containing 0.2 ml each of substrate and enzyme solution was incubated at 37 C for 5 min; the reaction was stopped by adding 0.5 ml of I2-KI solution, followed by measurement of the decrease in A at 620 nm upon addition of 2 ml H2O. One enzyme unit was defined as the enzyme activity causing 10% absorbance decrease at 620 nm under the stated assay condition.

Abbreviation: GA: gibberellin (gibberellic acid).
amylase (A, B, and C) (cf. Table II), amylose preparations were eluted from gel segments according to our previous method (18). Each respective enzyme solution was then added to 0.4% potato starch solution dissolved in acetate buffer (pH 5.3) and incubated up to 40 min. Aliquots withdrawn from the reaction mixture at 5-min intervals were subjected to the α- and β-amylase assay methods. From the results of these two analytical methods, we calculated the amount of maltose formed during the period in which the A at 620 nm decreased by one-half from its original value.

Isoelectric Focusing on Polyacrylamide Gel. To detect multiple forms of amylase, isoelectric focusing on the polyacrylamide gel was employed, basically following the method of Tanaka et al. (22). Following electrophoresis, zymograms were prepared by rinsing the gels thoroughly with 0.1 M acetate buffer (pH 5.3) and placing them on a glass plate coated with 0.4% hydrolyzed starch containing acrylamide gel. After incubation at 30 C for 20 to 30 min, the glass plate was immediately soaked in an I2-KI solution acidified with acetic acid. Amylase-bearing bands were decolored on a blue-brown background. For characterizing amylase bands A, B, and C, general properties of the enzyme preparations were tested according to the method of Jacobsen et al. (11). The crude enzyme preparation was either dialyzed overnight against 20 mM acetate buffer (pH 3.7) or heated at 70 C for 15 min in the presence of 10 mM CaCl2. The treated enzyme samples were applied to the gel isoelectric focusing to make zymograms. To examine the effect of metal ions on enzyme stability, the same enzyme samples were applied to the gels after being incubated with 1 M HgCl2 or 1 mM EDTA at 37 C for 1 hr. To examine the specificity of the amylase bands against different substrates, 0.4% amylopeptin or 0.4% β-limit dextrin-coated gel plates were used to make zymograms.

Starch Film Method. Rice seeds at selected germination stages were frozen with liquid N2, then sectioned with a Cryostat (Ames, Iowa). The sections of seeds obtained were placed directly on glass slides coated with a thin starch film and incubated at 25 C for 5 to 20 min (0.5 day, 20 min; 1 day, 10 min; 1.5–6 days, 5 min). Starch-coated microscope slides were prepared in a manner similar to that for making amylase zymograms (22). At the end of incubation, seed specimens were removed and the glass plate was immersed in an acidic I2-KI solution. Areas having amylase activity were decolorized on a blue-brown background.

RESULTS AND DISCUSSION

We first determined the time sequence changes of α- and β-amylase activities in each of the scutellum and endosperm samples during germination of rice seeds (Table I). On day 2, α-amylase activity in the endosperm, determined by the blue value method, was approximately seven times higher than that in the scutellum, whereas β-amylase activity in the endosperm, assayed by the reducing value method, was four times higher than that in the scutellum. As germination proceeded, enzyme activities in the endosperm continually increased, while those in the scutellum did not change greatly during germination; scutellum β-amylase activity showed a gradual decline.

We then examined the specific localization of amylase activities by means of the starch film technique. At an early stage of germination, about 12 hr after seed imbibition, amylase activity was almost exclusively localized in the epithelium (Fig. 1); after 1.5 days, the activity had spread progressively to the endosperm. Amylase activity in the aleurone layers was detectable only after day 2, and became intense at the 4- to 6-day stage. Inasmuch as essentially the same reaction patterns were obtained using the β-limit dextrin film as the substrate, it is likely that amylase molecules present in the epithelium at the very initial stage are of α-type.

To characterize the nature of amylase molecules produced during germination, it was necessary to compare the zymogram patterns between endosperm and scutellum. Results of gel isoelectric focusing, using extracts prepared from either scutellum or endosperm samples, clearly revealed nearly identical major bands (A, B, and C) in the two tissue extracts (Fig. 2). Mixing the two extracts did not alter the band patterns (data not shown). Some features merit description from the results of the zymogram analysis. In contrast to a relatively uniform enzyme pattern in the scutellum during the 2- to 6-day incubation (except for one additional band appearing below A on the 4th day) it was apparent that the endosperm tissue band C, together with several additional bands above band C, intensified in the late stages of germination. Our previous studies with endosperm tissues indicated that bands A and B are α-amylase while band C is β-amylase (18). It is evident from results shown in Table II that band C cannot attack β-limit dextrin, showing characteristic β-amylase property. The lower yield of reducing sugar (maltose) in bands A and B, in comparison with that in band C, indicates that the former two amylases are of the endo-type whereas the latter is of the exo-type.

We previously reported (22) that amylase patterns on isoelectric focusing gels were nearly the same between GA-treated embryoless rice seeds and the embryo-attached half-seeds. The present experiments show that in the very initial germination stage (12 hr after imbibition), amylase activities were almost exclusively localized in the epithelium, suggesting that enzyme biosynthesis in situ

Table I

<table>
<thead>
<tr>
<th>Germination (day)</th>
<th>Tissue</th>
<th>Amylase activity</th>
<th>α-amylase (unit × 10^3)</th>
<th>β-amylase (unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Scutellum</td>
<td></td>
<td>0.7</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>Endosperm</td>
<td></td>
<td>4.7</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>Scutellum</td>
<td></td>
<td>1.7</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Endosperm</td>
<td></td>
<td>18</td>
<td>66</td>
</tr>
<tr>
<td>6</td>
<td>Scutellum</td>
<td></td>
<td>0.6</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>Endosperm</td>
<td></td>
<td>21</td>
<td>73</td>
</tr>
</tbody>
</table>

Samples of 100 each of scutellum and endosperm tissues, dissected after 2, 4, or 6 days of germination, were homogenized as described in the text. Rice amylases were assayed for α- and β-amylase activities; data presented in the table are the averages of duplicate analyses.
is in this specific cell region. A massive amount of experimental data has accumulated that shows the production of $\alpha$-amylase to be induced by GA in the aleurone cells of barley seeds (5, 7, 9, 10, 11, 12). There is doubt concerning whether or not similar mechanism(s) operate in other germinating cereal seeds. Although it appears that GA occupies a crucial role in amylase formation in starchy seeds in general, some circumstantial evidence indicates that starch digestion begins from the site adjacent to the embryo (3, 23).

The previous research and our present results raise the following questions: (a) Are the scutellar amylase(s) identical to those from the endosperm? (b) Are the scutellar amylase(s) induced by GA? Concerning question (a), the present experiments strongly suggest the epithelium to be the site of initial enzyme formation in the embryo, and also indicate that the epithelium has a more important role in the hydrolytic digestion of starch reserve than do the aleurone cells. It is established that in the later germination stages amylase activities develop in the aleurone cells (Fig. 1). The newly formed amylase bands detectable above band C after 4 days on the gels (Fig. 2) may well reflect this additional enzyme activity. With regard to question (b), there is considerable experimental evidence showing the primary production of GA to be in embry-
Fig. 2. Comparison of amylase zymograms of crude extracts obtained from scutellum and endosperm tissues of rice seeds at various stages of germination. Crude scutellar extracts (100 μl each) and crude endosperm extracts (20 μl each) prepared at various germination stages were applied to gel for isoelectric focusing (pH 4–6). To make zymograms, gels were incubated with the starch gel-coated glass plates for 20 min. Other experimental details are described in the text.

Table II
Properties of scutellar amylases of germinating rice seeds

<table>
<thead>
<tr>
<th>Enzyme stability:</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>acid treatment (pH 3.7)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>heating (70°C, 15 min)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MgCl₂ (1 mM)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>KI (1 mM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Substrate susceptibility:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amylopectin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-limit dextrin</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Reducing power (mg maltose)</td>
<td>0.05</td>
<td>0.04</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Crude enzyme was prepared from scutellar tissue as described in the text. Enzyme properties in crude extracts were determined from the zymogram patterns of the gel electrofocused samples (cf. Fig. 2). In experiments testing enzyme stability, the symbol (+) indicates that the enzyme was resistant to various treatments, whereas (-) signifies that the enzyme activity was inhibited. In experiments to test substrate susceptibility, (+) signifies that the enzyme band appeared on the zymogram. Reducing power of amylase bands A, B and C, represents the amount (mg) of maltose molecules produced during the assay period, in which one half of the original soluble starch was hydrolyzed (see text).
onic tissues (14, 19, 20). It is likely that GA first takes part in amylase formation in the epithelium, later diffusing to aleurone cells where GA induces the de novo synthesis of additional amylase molecules (after the 2nd day).

In contrast to very thorough investigations on α-amylase formation in germinating cereal seeds, the nature of β-amylase biosynthesis and its role in the reserve starch digestion are relatively unexplored. In barley and wheat seeds, it has been demonstrated that β-amylase molecules exist in association with the protein in the endosperm tissues (21, 24). It is now found that β-amylase (band C) exists in the scutellum of germinating rice seeds; it is presumably synthesized in the epithelium. Since β-amylase activity was found to increase in the endosperm tissues as germination proceeded, it is intriguing to explore further the mechanism(s) of β-amylase formation and activation.

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LITERATURE CITED