Embryoless Wheat Grain

A NATURAL SYSTEM FOR THE STUDY OF GIBBERELLIN-INDUCED ENZYME FORMATION

Anwar A. Khan, Rita Verbeeck, Earl C. Waters, Jr., and Henry A. van Onckelen
New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456 and Laboratory of General and Biological Chemistry, State University of Gent, Gent, Belgium

ABSTRACT
Yorkstar wheat, grown in New York State, has a high percentage (10–11) of grains without embryos. The embryoless grains have viable aleurone layers and show no sign of injury. These grains are able to support α-amylase synthesis only in the presence of gibberellin A₃ (GA₃). In the absence of GA₃, some protein synthesis occurs in embryoless grains during the early hours of soaking, indicating that such activity occurs prior to and independent of GA₃ induction of α-amylase. The level of β-amylase on a dry weight basis is the same in embryoless and normal grains and decreases with time of soaking. In the presence of GA₃, β-amylase decreases at a slower rate. Isoenzymes of α-amylase from GA₃-treated embryoless and normal grains show qualitative as well as qualitative differences. Cycloheximide (60 μg/ml) completely inhibits the synthesis of α-amylase by embryoless grains. Of the RNA synthesis inhibitors, actinomycin D (60 μg/ml) was ineffective while 6-methylpurine (60 μg/ml) gave 65% inhibition without decreasing the number of isoenzymes.

The phenomenon of embryolessness in cereal grains, notably barley, wheat, and rye, has been described by a number of workers (10, 11, 14, 15, 24, 25, 27, 31). In some of these studies (11, 14, 15, 25) structures of the embryoless and normal grains are compared. According to Harlen and Pope (11), who first described this phenomenon in barley grains, embryolessness is probably a result of single fertilization. Based on a very limited study, they suggested that the fertilization from which the embryo would have formed failed to occur. These and other workers (14, 15, 25) found no evidence for the hypothesis that the development of the embryo had been arrested shortly after fertilization. The work in our laboratory showed that a soft white winter wheat (Triticum aestivum, cv. Yorkstar) grown in New York State contains in some lots up to 10 to 11% grains which lack embryos and scutella. This percentage is considerably higher than those reported previously from any other source.

The naturally occurring embryoless grain offers a distinct advantage over de-embryonated grain obtained by surgical means in studying embryo-related biochemical processes in cereal grains. The embryoless grain system is injury-free (11, 25). It, therefore, excludes the possibility of secondary metabolic changes that occur following excision of plant tissues (1, 6, 13, 19, 21, 26, 28, 30). It is reported that only a small damage to the aleurone of germinating barley decreases by 40 to 50% the production of α-amylase (3). Lyon (25) reported in 1928 that embryoless wheat grain was able to respire under conditions favorable for germination and that under these conditions there was evidence for amylase production. We report here on the ability of the embryoless grains to synthesize α-amylase and its isoenzymes. An attempt is also made to determine the nature of synthesis of various forms of α-amylase in these grains.

MATERIALS AND METHODS
The embryoless and normal wheat grains (Triticum aestivum, cv. Yorkstar, collected from Castile, N. Y., 1971 harvest) were carefully screened under a microscope. The average weights of a single embryoless and normal grain determined by weighing 200 grains were: embryoless grain, 31.0 mg; normal grain, 35.2 mg. Staining with tetrazolium showed that the aleurone cells from the embryoless and normal grains were equally sensitive to the stain. Microscopic examinations of the grain revealed no injury to the grain. Photomicrographs of sections of embryoless and normal grains are shown in Figure 1. There is no evidence of the presence of embryo or embryonic tissue in the embryoless grain. The region where the embryo is located in the normal grain (12) is designated by the notation “X” (Fig. 1). In both types of grain a single layer of aleurone can be seen extending to the embryo or the region where the embryo should have been in the embryoless grain. Seed coats are complete in both cases. The detailed structures of the embryoless wheat grain shown here resemble closely the structures of the embryoless barley grain (11).

Sterilized (1% sodium hypochlorite for 15 min) and washed embryoless and normal grains were incubated at 20 C in GA₃ solution in Petri plates for various lengths of time. In some experiments grains were transferred to inhibitors (60 μg/ml) at 0 and 8 hr after incubation, shaken for 2 hr at 4 C, and then returned to 20 C. Grains were washed and homogenized in 0.2% CaCl₂ in a Virtis 45 homogenizer for 1 min at full speed (45,000 rpm), and the homogenate was spun at 5,000g. The supernatant was assayed for α-amylase according to Briggs (2) using β-limit dextrin (obtained by treating 1.0% soluble starch with barley β-amylase from Fluka) as a specific substrate. The activity was expressed in ID₅ (iodine dextrin color) units. α-Amylase in the supernatant was concentrated by ammonium sulfate (75.0% saturation) precipitation and dialysis against

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Fig. 1. Longitudinal and transverse sections of mature, soaked (7.5-16 hr) Yorkstar wheat. A, B: Embryoless grain; C, D: normal grain. The limits of the aleurone layer (one cell thick) in the embryo region are shown by arrows. E: Starchy endosperm; F: furrow of grain; L: leaves of embryo; P: pericarp and seed coat; R: primary root of embryo; S: scutellum of embryo with a single layer of epithelial cells adjacent to the endosperm; X: site of embryo in embryoless grain. ×32.5. Labeled according to Hayward (12).

0.01 M tris-HCl, pH 7.5. α-Amylase isoenzymes were resolved by electrophoresis on polyacrylamide gels and detected by treatments with β-limit dextrin and I-KI solutions according to Van Onckelen and Verbeek (32).

β-Amylase in the 27,000g supernatant of grain extracts was assayed by the dinitrosalicylate method for reducing sugars (22). One milliliter of 1% starch solution in 0.0128 M acetate buffer, pH 5.3, and 0.008 M NaCl was treated with 1 ml of enzyme preparation at 25 C for 3 min at pH 5.3 before stopping the reaction by adding 2 ml of dinitrosalicylate reagent containing 0.04 g of dinitrosalicylic acid, 0.6 g of sodium potassium tartrate, and 0.032 g of NaOH. The mixture was boiled for 5 min and cooled, and the color was determined spectrophotometrically at 540 nm after addition of 20 ml of water. In all cases the pH of the enzyme was lowered to 3.4 with HCl then readjusted with NaOH to 5.3 to inactivate the α-amylase activity.

For the synthesis of labeled protein 5 grains per treatment were soaked on 0.75% Bacto agar with or without 1 μM GA₃ at 25 C and at various times incubated for 2 hr in 2.5 μCi of ³⁵C-leucine and 1 ml of 1 mm leucine. After rinsing with 0.1 mm leucine the grains were homogenized in a solution of 0.2 M NaCl and 0.1 mm leucine and spun at 12,000g for 10 min. Protein in an aliquot of the supernatant was precipitated with 12% trichloroacetic acid collected on glass fiber filters, and radioactivity was determined with a Nuclear-Chicago Unilux.
RESULTS AND DISCUSSION

The ability of the embryoless and normal grain to support $\alpha$-amylase synthesis in the presence and absence of GA$_3$ is shown in Figures 2 and 3. A lag period of about 20 to 24 hr is apparent before the onset of $\alpha$-amylase synthesis in barley embryoless grain in the presence of the hormone. In the absence of GA$_3$, the embryoless grain is unable to synthesize $\alpha$-amylase (Fig. 2). In the normal wheat grain the synthesis of $\alpha$-amylase in the presence of GA$_3$ is only slightly higher than that shown in the absence of the hormone (Fig. 3).

In order to determine whether injury plays a part in altering the level of $\alpha$-amylase in embryoless grain, small portions of the grain were excised where the embryo normally occurs. Excision did not reduce the response of the remaining grain to GA$_3$ on a unit weight basis.

The results clearly show a dependence of embryoless grain on GA$_3$ for the production of $\alpha$-amylase. However, the efficiency of GA$_3$-induced $\alpha$-amylase synthesis in the embryoless grain is still about one-third of that of the normal grain in 48 hr (Figs. 2 and 3). This evidently suggests that optimal synthesis of $\alpha$-amylase depends on factors made available to the aleurone by the embryo. It had been shown previously that GA$_3$ is unable to restore fully the $\alpha$-amylase-synthesizing capacity of the grain following excision of the embryo (29).

Contrary to the situation in the normally germinating barley grain, the injury does not appear, however, to have any influence on GA$_3$-induced $\alpha$-amylase in embryoless wheat grains (3). The reason for this is not known.

The time course of soluble protein synthesis in the embryoless grain in the presence of the hormone resembles that of $\alpha$-amylase (Fig. 4). In the absence of the hormone, however, there was an early increase in protein synthesis in this grain. This suggests that gibberellin action produces some proteolytic enzyme(s) in this system prior to the synthesis of $\alpha$-amylase. An early rapid synthesis of protein occurs in the normal grain with or without GA$_3$. This synthetic activity drops considerably in 48 hr; thereafter the synthesis levels off.

The levels of $\beta$-amylase in the normal and embryoless grains at various points in the soaking period are shown in Table I. It can be seen that the level of this enzyme on a dry weight basis at 0 hr is about the same in the two types of grains. After soaking, the level of enzyme decreases in both types of grains, although more slowly in the presence of GA$_3$.

The action of this hormone in reducing the rate appears to be more pronounced in the embryoless grain. The factors influencing the level of this enzyme were not studied. The data clearly show that the enzyme is already present in the grains. The action of GA$_3$ in reducing the rate of disappearance of the enzyme may be related to its action on secretion, leaching, or other processes. Furthermore, it is clear from these data that the embryo is not critical in the production and control of $\beta$-amylase during soaking of the grains. Similar conclusions were made in studies with barley grain (34). In view of this, the amylase activity reported by Lyon in 1928 (25) in the embryoless grain of wheat in the absence of GA$_3$ can be attributed to $\beta$-amylase.

![Fig. 2. $\alpha$-Amylase synthesis by embryoless wheat grain with and without GA$_3$ (1 $\mu$M) treatment. $\alpha$-Amylase is expressed in IDC units/gram·ml CaCl$_2$, 0.2%.

![Fig. 3. $\alpha$-Amylase synthesis by normal wheat grain with and without GA$_3$ (1 $\mu$M). Enzyme activity is expressed in IDC units/gram·ml CaCl$_2$, 0.2%.

![Fig. 4. Protein synthesis by embryoless (- embryo) and normal (+ embryo) wheat grains in the presence and absence of GA$_3$ (1 $\mu$M).

Table I. $\beta$-Amylase Activity in Embryoless and Normal Grains Soaked with and without GA$_3$, 1 $\mu$M

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme Activity after Soaking for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Embryoless</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>0.696</td>
</tr>
<tr>
<td>GA$_3$</td>
<td>0.461</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>0.649</td>
</tr>
<tr>
<td>GA$_3$</td>
<td>0.497</td>
</tr>
</tbody>
</table>

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means, however, ABA inhibition of GA3-induced a-amylase synthesis is not reversed by kinetin (16). In view of this, it was considered desirable to test the effect of a cytokinin and ABA on the embryoless grain. ABA inhibited the GA3-induced a-amylase production in the embryoless grain. This inhibition was not reversed by kinetin. This result gives further credence to the earlier hypothesis that the embryo or some factor associated with it is essential for cytokinin reversal of ABA inhibition of a-amylase synthesis (16, 35).

In order to elaborate further on the differences and similarity in the normal and embryoless grains, the effect of GA3 on a-amylase isoenzymes was studied in the two types of grains. The zymograms of a-amylase isoenzymes at various points of soaking of the two types of grains are shown in Figure 5. It can be seen that 9 isoenzymes are resolved in the embryoless grain in response to GA3 in 24 hr. This number remains constant until 48 hr. In the case of normal grain the numbers of isoenzymes in 24 hr in the absence and presence of GA3 are 5 and 8, respectively. In 72 and 144 hr, the normal grains have 9 and 12 isoenzymes, respectively, with GA3 having no visible effects. The results indicate some qualitative differences in the a-amylase-producing potential of the embryoless and normal grains, at least during early periods of soaking. Three additional isoenzymes in the normal grain appear after 144 hr. This increase could be a result of an interplay of additional factors supplied by the growing embryo with the enzyme-synthesizing machinery in the aleurone layer (16, 35). An increasing number of isoenzymes of a-amylase with increasing periods of soaking has been reported previously in the germinating barley grain (32).

In order to determine the nature of gibberellin-induced formation of isoenzymes in the embryoless grain, specific inhibitors of nucleic acid and protein synthesis were used at two different times of incubation of the grain. Cycloheximide completely inhibited a-amylase formation and its isoenzymes, while 6-methylpurine caused a 65% loss of a-amylase activity without noticeably decreasing the number of isoenzymes (Table II). Actinomycin D at the concentration used (60 \(\mu\)g/ml) had no inhibitory effect on a-amylase or its constituent isoenzymes. This concentration of actinomycin D was perhaps lower than that required to inhibit the enzyme synthesis. It has been suggested that the inability of actinomycin D to inhibit a-amylase synthesis in aleurone cells at low concentrations could be due to its ineffectiveness in blocking RNA synthesis (4) or to its varying effects during the lag period of enzyme synthesis (9). Other inhibitors of RNA synthesis, including 6-methylpurine, however, are known to inhibit a-amylase synthesis (4, 5, 33). On the basis of the data presented here, and those of others, it is reasonable to suggest that the enzyme is synthesized de novo in the embryoless grain and that the enzyme has a requirement for RNA. The extent or manner of RNA participation in a-amylase synthesis, however, cannot be deduced from the present data. Nor can it be concluded that the action of 6-methylpurine on a-amylase synthesis is at the transcriptional level. All nine isoenzymes are present in the embryoless grain in spite of 65% inhibition by 6-methylpurine. This quantitative inhibition could be envisaged as occurring because of changes at the transcriptional, translational, or other levels. It is interesting to note in this connection the recent demonstration by Gibson and Paleg (8) that a-amylase in wheat aleurone is an extracellular lysosomal enzyme. Implicit in this is the suggestion that a-amylase formation is not absolutely dependent on nucleic acid synthesis.

The results outlined here clearly show that the naturally occurring embryoless grain is an attractive and effective means for studying the changes induced by an embryo-based hor-

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**Table II. Influence of Inhibitors (60 \(\mu\)g/ml) on a-Amylase Induction by GA3 in 'Embryoless' Wheat**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>a-Amylase Activity</th>
<th>No. of Isoenzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA3 1(\mu)M</td>
<td>64.5</td>
<td>9</td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>68.2</td>
<td>9</td>
</tr>
<tr>
<td>+ Actinomycin D&lt;sup&gt;2&lt;/sup&gt;</td>
<td>72.5</td>
<td>9</td>
</tr>
<tr>
<td>+ 6-Methylpurine&lt;sup&gt;1&lt;/sup&gt;</td>
<td>22.1</td>
<td>9</td>
</tr>
<tr>
<td>+ 6-Methylpurine&lt;sup&gt;2&lt;/sup&gt;</td>
<td>29.2</td>
<td>9</td>
</tr>
<tr>
<td>+ Cycloheximide&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ Cycloheximide&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>1</sup> Inhibitors applied at 0 hr.
<sup>2</sup> Inhibitors applied after 8 hr of incubation.
AMYLASES IN EMBRYOLESS WHEAT GRAIN


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LITERATURE CITED