Gibberellic Acid Controlled Synthesis of α-Amylase in Barley Endosperm [1]

J. E. Varner

RIAS, 7212 Bellona Avenue, Baltimore 12, Maryland

Introduction

Because the endosperm half of a barley seed produces several hydrolytic enzymes in response to added gibberellins (1, 4, 5, 8), it is an attractive experimental system for a study of the mechanism of action of gibberellic acid. The only living cells in the half seed are those of the aleurone layers. These are an apparently homogeneous collection of respiring, nondividing cells which have the highly specialized function of producing and releasing some of those enzymes which are required to digest the starchy endosperm in preparation for its utilization by the growing embryo.

Because the work of Paleg (5) had shown that the increase in α-amylase activity in barley endosperm halves is absolutely dependent upon added gibberellic acid and because α-amylase is heat stable and easily purified, a detailed study of the gibberellic acid triggered increase in α-amylase activity has been initiated.

Methods and Materials

The barley seeds (*Hordeum vulgare*, var. Himalaya) were cut in half on the equatorial axis and the embryo-half discarded. The endosperm halves were soaked in Chlorox (a commercial bleach containing 5 % sodium hypochlorite) diluted five-fold with distilled water for 15 to 20 minutes, rinsed in sterile distilled water and aseptically transferred to sterile moist sand in petri dishes. After 3 to 5 days at 17 to 23° (We call this period "preincubation." Within the limits indicated, there is no detectable influence of variations of time and temperature on the capacity of the half-seeds to respond to gibberellic acid added after the preincubation.), 10 half-seeds were transferred to a sterile 25-ml Erlenmeyer flask containing 2.0 ml to 1.0 μM sodium acetate buffer (pH4.8) and the appropriate treatment solution. The flasks were shaken at top speed for 18 to 20 hours at 25° on a Dubnoff metabolic shaker. The medium was poured off and the half-seeds rinsed once with 3.0 ml of distilled water. The half-seeds were ground in a mortar with sand and 5.0 ml 0.001 M acetate buffer (pH 4.8). After centrifugation at 1000 × *g* the medium and extract were assayed separately by the method of Shuster and Gifford (7). The assay was put on a quantitative basis by the use of crystalline α-amylase prepared according to Schwimmer and Balls (6).

Dissection of the half-seeds into aleurone layers plus seed coat and starchy endosperm was performed after the 3-day preincubation.

For in vivo incorporation of labeled phenylalanine into barley proteins 10 μc of L-phenylalanine-C^{14} (6 μc per μmole, California Corporation for Biochemical Research) was added to each flask at the start of the incubation. In each case, the sample was dialyzed against a solution containing 0.015 M Tris (pH 7.8), 0.01 M $CaCl_2$ and carrier L-phenylalanine before addition to a DEAE column (1 × 20cm) and eluted with a linear gradient formed with 200 ml of solution containing 0.015 M Tris (pH 7.8) and 0.01 M $CaCl_2$ and 200 ml of solution containing 0.015 M Tris (pH 7.8), 0.01 M $CaCl_2$ and 0.5 M NaCl. The fractions were about 10 ml.

Results and Discussion

Visual inspection of the gibberellic acid treated half-seeds indicated that that part of the starchy endosperm in contact with the aleurone layers was first dissolved and that the α-amylase was therefore probably coming from the aleurone layers. This was confirmed quantitatively (table I). This conclusion is

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Table I

Tissue Origin of α-Amylase

The gibberellic acid concentration was 10^{-6} M. Incubation was carried out at 20°. Other conditions were as described in Methods.

Tissue	— Gibberellic acid		+ Gibberellic acid	
	Medium	Extract	Medium	Extract
	μg	μg	μg	μg
Aleurone layers and seed coat	10	24	19	64
Starchy endosperm	7	4	7	2
Aleurone layers with seed coat and starchy endosperm	13	4	41	41

Table II

Factors Affecting α-Amylase Formation

The experimental details are described in Methods. The gibberellic acid concentration was 10^{-6} M.

Treatment	Medium	Extract	Total
	μg	μg	μg
— Gibberellic acid	0	4	4
Control	80	65	145
Control (anaerobic)	5	8	13
+ Dinintrophenol 10^{-3} M	0	8	8
" 10^{-4} M	3	12	15
" 10^{-5} M	24	24	48
+ *p*-fluorophenylalanine 10^{-4} M	28	26	54
" 10^{-5} M	28	47	75
" 10^{-6} M	82	80	162
+ Chloramphenicol (1 mg/ml)	10	46	56

in agreement with the recent report of MacLeod and Millar (4). Haberlandt (3) and Brown and Escombe (2) also concluded that the α-amylase in the endosperm of germinating barley was released by the aleurone layers.

The q_{O_2} (μl O_2 • $hour^{-1}$ • 100 mg fr wt^{-1}) for the aleurone layers still attached to the starchy endosperm, as measured in the Warburg respirometer at 25°, is 15. It is 0 for the isolated starchy endosperm, and 30 to 40 for the aleurone layers (plus seed coat) when separated from the starchy endosperm. The reason for the higher q_{O_2} for the isolated aleurone layers is not known. The aleurone layers (plus seed coat) contain 2.98 % N on a dry weight basis. The wet weight of the aleurone layers (plus seed coats) from 10 half-seeds is 168 mg and the dry weight is 80 mg.

Aerobic respiration (table II) and presumably

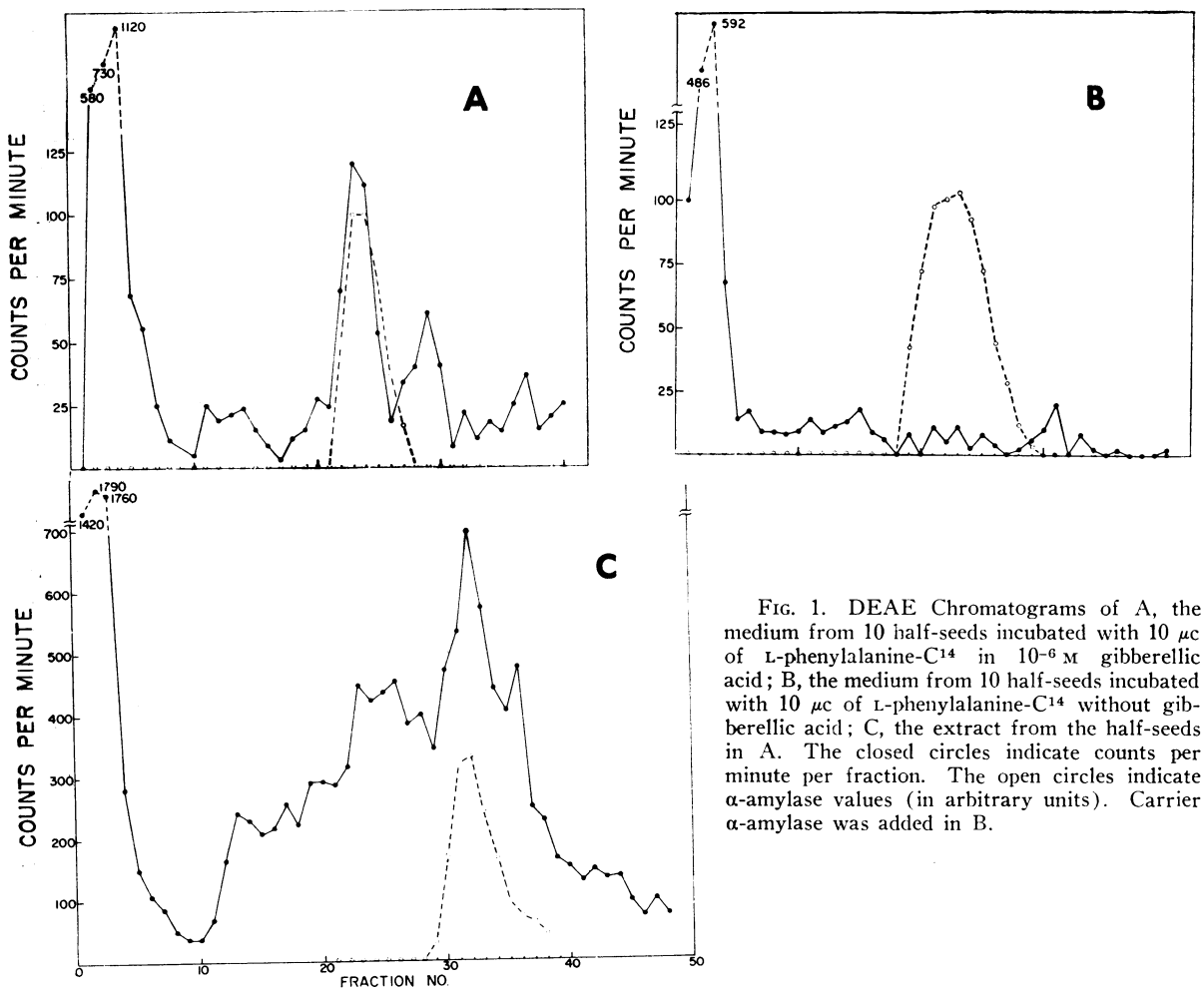

FIG. 1. DEAE Chromatograms of A, the medium from 10 half-seeds incubated with 10 μc of L-phenylalanine-C^{14} in 10^{-6} M gibberellic acid; B, the medium from 10 half-seeds incubated with 10 μc of L-phenylalanine-C^{14} without gibberellic acid; C, the extract from the half-seeds in A. The closed circles indicate counts per minute per fraction. The open circles indicate α-amylase values (in arbitrary units). Carrier α-amylase was added in B.

oxidative phosphorylation are necessary for α-amylase production. These requirements become understandable in view of the further finding (table II) that *p*-fluorophenylalanine and chloramphenicol inhibit formation of α-amylase. At this point it seemed worthwhile to check directly the possibility that the formation of α-amylase was the result of de novo synthesis. Accordingly the half-seeds were incubated in C^{14}-labeled phenylalanine and the incubation medium and the extract of the half-seeds separately analyzed by DEAE chromatography. Not only is α-amylase labeled (fig 1) but it also constitutes a major fraction of the radioactivity incorporated into proteins of the half-seeds. This labeled α-amylase is now being purified in preparation for partial hydrolysis (by proteolytic digestion) and fingerprinting to determine whether the entire α-amylase molecule is produced by de novo synthesis or whether the labeling results from addition to and modification of a precursor, as seems to be the case for the formation of α-amylase by resting *B. subtilis* cells (9). In any event the rate of respiration observed is more than adequate to support de novo synthesis of the α-amylase produced.

If one supposes that the observed synthesis of α-amylase requires a source of ATP and GTP and the participation of activating enzymes, ribosomes, messenger RNA, shuttle RNA and releasing enzymes according to the current theories of protein synthesis, which of the many possible steps is controlled by gibberellic acid? Results of experiments designed to show a gibberellic acid dependent synthesis of RNA have been equivocal. When azaguanine, thiocytosine, thiouracil, or actinomycin D are added to preincubated half-seeds at the same time as the gibberellic acid, there is no inhibition of α-amylase formation. When half-seeds which have been preincubated on sand for 3 days are further preincubated with these inhibitors for 24 hours before adding gibberellic acid, α-amylase formation is not inhibited. However, if the half-seeds are preincubated from the air dry state on sand moistened with the inhibitors, all of the inhibitors are effective in preventing α-amylase formation. This effect is reversible, i.e., when the inhibited half-seeds are further incubated with gibberellic acid in fresh buffer solution free of inhibitors, α-amylase is formed as usual. It is clear that there must be some RNA synthesis, including DNA-directed RNA synthesis, before the aleurone cells can respond to added gibberellic acid. It is not certain whether this RNA synthesis occurs before or after addition of the gibberellic acid.

While this work was in progress, Briggs (1) published evidence which shows that gibberellic acid enhances de novo synthesis of unidentified proteins by the aleurone cells.

Summary

The α-amylase produced by barley endosperm in response to added gibberellic acid is produced in the aleurone layers by de novo synthesis.

Acknowledgments

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