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THE BIOASSAY OF GIBBERELLINS*

R. L. JONES** and J. E. VARNER
MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Michigan

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Summary. A bioassay is described which is dependent upon the fact that gibberellin induced α-amylase release from barley half-seeds is proportional to the logarithm of gibberellin concentration applied. This bioassay has been successfully applied to the estimation of gibberellin-like substances in plant extracts. The bioassay has the following advantages: 1) Release of α-amylase is one step closer to the primary site of action of GA; 2) release of α-amylase is not affected by solvent residues and is apparently completely specific for gibberellin; 3) release of α-amylase is not affected by substances other than gibberellins present in crude plant extracts.

Gibberellic acid (GA_3) and other gibberellins enhance the release of reducing substances from barley endosperm (PALEG, 1960; YOMO, 1960). The quantity of reducing substances released is proportional to the logarithm of gibberellin concentration applied (PALEG *et al.*, 1964; NICHOLLS and PALEG, 1964). This proportionality has provided a basis for the estimation of gibberellins in plant extracts (COOMBE and COHEN, 1964). However, in our hands, this bioassay has proved erratic, perhaps because traces of a wide variety of substances present as impurities in the organic solvents used to prepare the plant extracts cause the release (or interfere with the measurement) of reducing substances from barley endosperm (BRIGGS, 1966).

The release of reducing substances is primarily a result of the gibberellin-dependent synthesis and secretion of α-amylase by the aleurone layers (YOMO, 1960; VARNER and CHANDRA, 1964). Thus, the production of α-amylase is one step nearer to the primary site of action of gibberellin. Therefore, measurement of α-amylase activity as a gibberellin bioassay may be expected to be less susceptible to non-specific interferences. Direct measurement of the α-amylase released by the barley endosperm has proved to be a simple, reproducible bioassay which is insensitive to solvent residues.

Procedure

Seeds of *Hordeum vulgare* (cv. 'Himalaya'), 1963 harvest, were cut in half transversely and the embryo portion discarded. The endosperm

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** Present address: Department of Botany, University of California, Berkeley, California 94720 (USA).

halves were sterilized by soaking in a 1% sodium hypochlorite (commercial bleach) solution for twenty minutes, followed by three washings in sterile water. Sterilized seeds were imbibed on sterile sand in 10-cm (4-inch) Petri dishes each containing 100 gm ignited sand moistened with 20 ml sterile water. Following a 3-day imbibition period, 10 half-seeds each were transferred to 25 ml Erlenmeyer flasks containing two micromoles of acetate buffer (pH 4.8), 20 micromoles of calcium chloride and the solution to be tested, in a final volume of 2 ml. The flasks containing the calcium chloride and buffer were autoclaved prior to commencement of the incubation period. As an added precaution against microbial contamination, 20 μg of chloramphenicol can be added to each flask. Incubation of the half-seeds was continued for 24 hours at 25° with continuous shaking at 40 oscillations/minute.

Following the incubation period, the medium was decanted into 100×7.5 mm centrifuge tubes and washings totalling another 3 ml were added. After this, the tubes were centrifuged for 10 minutes at $2{,}000 \times g$.

The assay for α-amylase was accomplished using suitable volumes (0.02—0.2 ml) of the supernatant fraction of the medium together with sufficient water to make a total volume of 1.0 ml. The reaction was started by adding to the medium 1.0 ml of the starch substrate and allowed to continue for a suitable period of time (1—10 minutes). The reaction was stopped by addition of 1.0 ml of iodine reagent. To this final reaction mixture, 5.0 ml of distilled water was added, mixed, and the optical density (O.D.) read at 620 mμ.

The decrease in O.D. at 620 mμ is directly proportional to the quantity of α-amylase present in the reaction mixture. The most accurate results are obtained by adjusting the enzyme aliquot and the time so that the optical density of the starch-iodine complex is about half the optical density of the zero-time control. Optical density values are converted to micrograms α-amylase released using a factor which is obtained for each particular starch sample by incubation with a pure α-amylase preparation:

$$\mu g\ \alpha\text{-amylase} = \frac{\Delta \text{O.D.} \times T_v \times \text{C.F.}}{t \times v}$$

T_v = volume of supernatant
ΔO.D. = O.D. of zero-time control *minus* O.D. of sample
C.F. = conversion factor for starch sample
t = time of incubation with starch
v = volume of supernatant taken for incubation

Preparation of Starch and Iodine Reagents. The starch solution for α-amylase assay was prepared from 150 mg of native (not solublized) potato starch, 600 mg KH_2PO_4 and 200 μM calcium chloride in a total volume of 100 ml of distilled water. The mixture was boiled for one

minute, cooled, then centrifuged for 10 minutes at 3,000 × g. The clear supernatant was decanted off and used for assay.

The iodine stock solution was prepared by mixing 6 gm of potassium iodide and 600 mg of iodine in 100 ml of water. One ml of the stock solution was added to 0.05 N hydrochloric acid to give a final volume of 100 ml. This is used to stop the α-amylase reaction.

Experimental Results

The amount of α-amylase released from barley half-seeds in response to GA_3 application is proportional in the range of 0.0005 μg/ml to

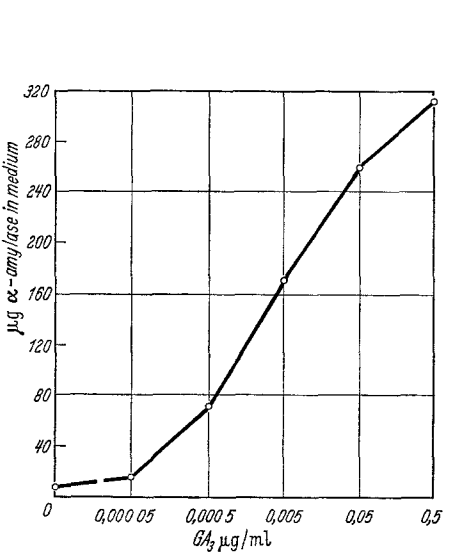

Fig. 1

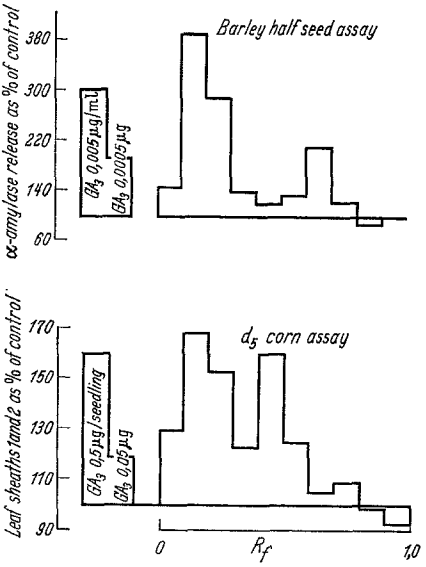

Fig. 2

Fig. 1. Relationship between gibberellic acid (GA_3) concentration and α-amylase release from barley half-seeds

Fig. 2. Barley half-seed and d_5 corn mutant assays of extracts of 20 *Pharbitis nil* seeds. Extracts chromatographed on a thin layer of Silica gel H and developed in a mixture of chloroform/ethyl acetate/acetic acid (60:40:5 v/v)

0.05 μg/ml (Fig. 1). Other gibberellins were also tested in this system (Table 1). Gibberellins A_1, A_4 and A_7 were found to be comparable to GA_3 with respect to α-amylase release; GA_5 on the other hand was considerably less effective. As expected, the pattern of response to the various gibberellins is similar to that for sugar release from barley half-seeds (Paleg *et al.*, 1964).

This method of measuring gibberellin-induced α-amylase release is also suitable for the bioassay of gibberellin-like substances present in plant

Table 1. *Potency of different gibberellins, expressed as percentage of GA_3-activity*

Gibberellin	Concentration µg/ml			
	0.0005	0.005	0.05	0.5
Gibberellic acid (GA_3)	100	100	100	100
GA_1	80	100	100	100
GA_7	70	73	78	80
GA_4	74	70	82	80
GA_5	10	10	40	65

extracts and agar diffusates (Fig. 2). An extract of 20 *Pharbitis nil* seeds known to contain gibberellin-like substances was chromatographed on a thin layer of silica gel. Following development of the chromatogram, the gel was divided into 10 equal portions corresponding to R_f 0.0—1.0, and each zone was eluted with water-saturated ethyl acetate. The eluates were reduced to dryness *in vacuo*, redissolved in 1 ml of acetate buffer (pH 4.8) and incubated with half-seeds. Although the extract contains substances which markedly inhibit lettuce root and hypocotyl growth, substantial α-amylase release was seen in fractions containing gibberellin-like substances. A similar number of *Pharbitis* seeds were also extracted and the chromatographed extracts assayed with the d_5 corn (*Zea mays*) mutant. The pattern of biological activity obtained by the corn-mutant assay resembled that obtained by measuring α-amylase release, except that biological activity due to a GA_5-like substance was absent from the α-amylase assay.

Table 2. *Effect of ethyl acetate and methanol residues on α-amylase. Release from barley half-seeds*

Treatment	µg α-amylase in medium
Water control	3.0
GA_3 0.05 µg/ml	78.3
GA_3 0.005 µg/ml	25.0
Ethyl acetate 500 ml 1.*	4.0
Ethyl acetate 500 ml 2.**	3.2
Ethyl acetate 250 ml 1.*	1.9
Ethyl acetate 250 ml 2.**	1.6
MeOH 250 ml 1.*	1.6
MeOH 250 ml 2.**	4.0

* Residue following initial evaporation of "Reagent" grade solvent.

** Distillate from initial evaporation re-distilled.

The release of α-amylase from barley half seeds is thought to be specific to gibberellins, in contrast to sugar release which is affected by residues present in certain solvents (BRIGGS, 1966). Briggs showed that the residue present following evaporation of 500 ml of ethyl acetate caused significant sugar release from barley half seeds. Similar experiments were performed to determine whether solvent "tails" affected α-amylase release. The results are summarized in Table 2. As can be seen, the residues had no effect on α-amylase release from barley endosperm pieces.

Discussion

The measure of gibberellin-induced α-amylase release from barley half seeds provides a suitable method for the bioassay of gibberellin-like substances. The induction of α-amylase synthesis and its subsequent secretion is specific to gibberellins; neither auxins nor cytokinins have any effect in this system (M. J. CHRISPEELS, personal communication). It is, however, inhibited by various naturally occurring growth inhibitors. CHRISPEELS and VARNER (1966) have shown that abscisin II can inhibit the effect of gibberellin in inducing α-amylase synthesis, although, on a molecular basis, approximately ten times as much abscisin is required to inhibit the GA-induced response. As abscisin has been found in several plant extracts (CORNFORTH *et al.*, 1966), the preparation of dilution series would appear necessary to obtain truly quantitative data on a bioassay of plant extracts for gibberellins.

Another source of error in the measurement of α-amylase released from half-seeds may arise from the presence of microbial contaminants in the surrounding medium. In contrast to the sugar-release assay described by PALEG *et al.* (1964), where the presence of microorganisms may amplify sugar release, microorganisms denature and destroy α-amylase. Thus measurement of α-amylase released never yields a false positive test for gibberellins. However, microbial contamination is seldom a problem if, as indicated in the Methods section, aseptic procedures are used in the preparation of both the seeds and medium. Addition of chloramphenicol at 20 μg/ml effectively inhibits bacterial growth and, at this concentration, has no known effect on plant metabolism (WILSON, 1966).

Table 3. *Effect of ethanol on α-amylase release from barley half seeds*

Treatment	α-amylase in medium μg
Water control	<10
Water + 1% EtOH	<10
GA_3 10^{-6} M	312
GA_3 10^{-6} M + 1% EtOH	120
GA_3 10^{-6} M + 2% EtOH	36

Residues from organic solvents have no effect on α-amylase release. This suggests that the reducing substances released by organic solvent residues must be a result of hydrolysis by an enzyme other than α-amylase, possibly β-amylase. This suggestion is strengthened by the fact that ethanol at a concentration of 5% caused significant release of reducing sugars (BRIGGS, 1966) while α-amylase release is inhibited by a concentration of 2% ethanol (Table 3).

Although all the experiments described above were performed with one variety of barley seed, other cereal seeds have been shown to produce α-amylase when treated with GA_3 (MORO *et al.*, 1960; OGAWA and IMAMURA, 1965). The choice of harvest from which barley seeds are taken has been shown to be important. Newly harvested seeds contain sub-

stantial levels of endogenous gibberellin which give rise to high "background" levels of α-amylase. Also, the amount of α-amylase released is greater from aged than that from newly harvested barley seeds. It may often prove useful to check a number of samples of commercially available barley and then secure an adequate supply of the optimal one, *i.e.*, having lowest "background" and highest α-amylase release.

Five gibberellins were tested for biological activity in this system, namely A_1, A_3, A_4, A_5 and A_7, and of these, GA_5 is the least effective in stimulating α-amylase release. Because the assay is much less sensitive to GA_5, a gibberellin frequently found in plant extracts (West and Phinney, 1959; Macmillan *et al.*, 1960), the method should be used either in conjunction with a bioassay system which is known to be sensitive to a wider spectrum of gibberellins (for example, dwarf corn mutants or lettuce hypocotyl), or after an initial screening with such a bioassay.

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R. L. JONES
Department of Botany,
University of California
Berkeley, California 94720 (USA)

J. E. VARNER
MSU/AEC Plant Research Laboratory,
Michigan State University
East Lansing, Michigan 48823 (USA)