Similarities Between Gibberellins and Related Compounds in Inducing Acid Phosphatase and Reducing Sugar Release From Barley Endosperm

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Abstract. Barley endosperm halves release acid phosphatase in response to several gibberellins and gibberellin precursors. Seed halves incubated with $10^{-7}$ M GA$_3$ at $29^\circ$ begin to release phosphatase after 11 hr and release it for another 26 hr in response to GA$_4$. After 37 hr, the rate of release slows to that of seed halves incubated without GA$_3$. GA$_3$ is active at $10^{-10}$ M and maximally active at $10^{-7}$ M. Comparative activity of 12 gibberellins and gibberellin precursors is GA$_1$ = GA$_2$ > GA$_3$ > GA$_4$ = GA$_5$ > GA$_6$ > GA$_7$ > GA$_8$ = GA$_9$ > GA$_10$ > GA$_14$ > GA$_b$ = GA$_g$ > (-)kaurenoic acid > (-)kaurene. These compounds show the same order of activity and approximately the same relative activity in inducing reducing sugar release as in inducing phosphatase activity. The activity of each compound increases with its presumed position in a biosynthetic pathway leading from kaurene to GA$_3$. This correlation suggests that activity may be a reflection of the efficiency of conversion to an active form within the seed half.

The ease of measuring the acid phosphatase response and the sensitivity of the seed halves to low concentrations of many gibberellins and gibberellin precursors makes this a useful bioassay for gibberellin-like substances.

During germination, the barley embryo releases gibberellin-like substances which induce the aleurone to release several hydrolytic enzymes (4). These enzymes subsequently degrade the starchy endosperm to provide carbon and energy sources for the growing embryo. Exogenous gibberellin will substitute for the embryo in causing enzyme release (12, 13, 17) and the biochemistry of the response has been studied extensively with respect to $\alpha$-amylase, ribonuclease and protease (3, 7, 15).

Acid phosphatase is also secreted in response to gibberellin, although studies of this response are relatively limited. MacLeod and coworkers (11) found that acid phosphatase is the first of 4 enzymes to be secreted in response to GA$_3$, followed by endo-$\beta$-glucanase, $\alpha$-amylase and protease. They found a relatively high concentration of acid phosphatase within the untreated seed which was not secreted in the absence of GA$_3$. More recently, Pollard and Singh (14) reported on the secretion of specific acid phosphatases following GA$_3$ treatment of isolated barley aleurone and confirmed that phosphatase release is a relatively early effect of GA$_3$ treatment.

It is of interest to compare the activity of different hormones in inducing phosphatase release and to compare the phosphatase response with other gibberellin-induced endosperm responses. In this report, the effect of 12 gibberellins and gibberellin precursors on total acid phosphatase release from embryoless barley seed halves is described, and phosphatase release due to these compounds is compared with reducing sugar release. In addition, a gibberellin assay based on phosphatase release is described and compared with the barley endosperm reducing sugar assay and the dwarf-3 maize assay.

Materials and Methods

Seed Preparation and Incubation. Barley seed halves (Hordeum vulgare L., cv. White Naked Atlas) were prepared and incubated in a manner similar to that described by Paleg et al. (13) for the barley endosperm reducing sugar assay. Embryoless halves were sterilized in 1 % sodium hypochlorite for 20 min and rinsed 10 times with distilled water. Groups of 3 seed halves were incubated in 1.0 ml of a solution of streptomycin sulfate (100 $\mu$g/ml) and hormone. Incubations were carried out in sterilized 6-dram (25 mm diameter, 70 mm height) screw top vials. At the end of the incubation period, seed halves were removed from the incubation liquid and the liquid was frozen until assays were performed. Freezing caused no detectible loss of activity. Failure to remove seed halves before freezing caused increased phosphatase activity in the liquid, possibly resulting from cell disruption.

Acid Phosphatase Assay. Frozen incubation liquids were thawed and diluted to 5.0 ml with distilled water. One ml portions were mixed with
1.0 ml portions of substrate solution containing $7 \times 10^{-4}$ mmole $p$-nitrophenylphosphate (disodium salt) (PNPP) and the mixtures were incubated for 30 min at 35°. The solutions were then made alkaline with 5.0 ml 0.1 N NaOH and the absorbancy at 410 nm was determined. Absorbancies were compared to a $p$-nitrophenol standard to determine the amount of PNPP hydrolyzed during the enzyme incubation.

The substrate solution was prepared by mixing 30 ml acetate buffer (pH 4.8, 0.1 M), 10 ml PNPP (50 mg/100 ml $H_2O$) and 1.0 ml 0.1 M $MgCl_2$.

**α-Amylase Assay.** α-Amylase was assayed according to the method described by Jones and Varner (9).

**Reducing Sugar Measurement.** Incubation liquids were shaken with 0.5 g Amberlite IR-120 (H+) resin (13) and portions of the liquid tested for their ability to reduce 3,5-dinitrosalicylic acid (1). Reduced dinitrosalicylic acid was measured at 540 nm and the measurements were converted to maltose equivalents.

**Dwarf Maize Leaf Sheath Elongation Assay.** Homozygous dwarf-3 segregants of Zea mays L. were used as described previously (8).

**Preparation of Gibberellin Extracts.** Gibberella fujikuroi (Saw.) Wrl., strain Lilly M119, was grown for 2 weeks on potato dextrose broth at laboratory temperature. The mycelium was filtered from the liquid and ethyl acetate-soluble organic acids extracted from the filtrate (8). Extracts were chromatographed on thin layers of silica gel G in benzene:n-butanol:acetic acid (85:15:5; v/v/v) (10). Twelve zones of the adsorbent, including 10 Rf zones, plus a zone below the origin and a zone above the front, were scraped from the plate and washed 3 times with 3 ml distilled acetone. The eluates from each zone were combined and the solvent evaporated to dryness. The residue was redissolved in 5 ml hot (55°) water and a portion of the solution assayed for gibberellin-like activity.

**Results**

The sensitivity of the response of barley half seeds to many gibberellins and gibberellin precursors makes acid phosphatase release useful as an assay for these substances. Ethyl acetate-extractable organic acids from Gibberella fujikuroi were chromatographed and fractions from the chromatogram compared in the barley endosperm reducing sugar assay, the dwarf-3 maize assay and for the ability to induce acid phosphatase release from seed halves.

The results of these assays are shown in Fig. 1. Three regions of activity are seen in the phosphatase and reducing sugar tests and 2 in the maize assay. The activity from zones 2 and 3 corresponds in position to $GA_3$, $GA_2$, and $GA_1$, while the activity from zones 5 and 6 corresponds in position to $GA_4$ and $GA_7$. Slight activity in barley assays from zone 10 could be due to $GA_9$ and other relatively non-polar gibberellins. Zones below the origin and above the front were inactive, showing that solvents and adsorbent contain no active material.

The effect of temperature on acid phosphatase activity induced by $10^{-7}$ M $GA_3$ is shown in Fig. 2. Maximum activity occurs from seeds incubated at 29° and diminishes rapidly at temperatures above 30°. Phosphatase activity appears after 11 hr incubation in $10^{-7}$ M $GA_3$ and increases rapidly for about 20 hr (Fig. 3). After about 38 hr, seed halves incubated without $GA_3$ begin to release phosphatase spontaneously and the level of the enzyme in the incubation liquid increases steadily for the duration of the test. During the period of spontaneous release in water controls, $GA_3$-treated seed halves continue to release phosphatase, but at a rate equal
to the controls. α-Amylase and reducing sugar release was also measured for comparison, and found to begin after about 24 hr incubation. Based on these observations, tests were carried out at 29° for approximately 40 hr to insure maximum phosphatase release with a minimum of phosphatase activity in the water controls.

Acid phosphatase is released in response to a wide range of GA₃ concentrations (Fig. 4). Significant activity is caused by 10⁻¹⁰ M GA₃ (3.5 × 10⁻⁶ µg/ml) and maximum activity occurs at 10⁻⁷ M GA₃.

Table I shows a comparison of the activity of 4 concentrations of gibberellins and gibberellin precursors in inducing phosphatase and reducing sugar activity. Incubation liquids from 10⁻⁶ and 10⁻⁷ M gibberellin were tested for α-amylase activity and it was found that only GA₁, GA₂, GA₃, GA₄ and GA₇, the 5 most active compounds in the phosphatase and reducing sugar tests, caused detectible levels of amylase activity. The presence of reducing sugars in incubation liquids from most of the remaining compounds suggests that amylase had been present but was inactive at the time of the assay.

The order of activity of the 12 compounds is the same in the reducing sugar and phosphatase tests. In the case of 10⁻⁸ M kaurene and 10⁻⁷ M GA₁₃, phosphatase was released without concomitant reducing sugar release. This suggests that phosphatase measurement is a more sensitive test of gibberellin-like activity than the reducing sugar test.

**Discussion**

Paleg et al. (13) reported GA₁, GA₃, and GA₄ to be most active, GA₅ and GA₆ to be less active and GA₇ and GA₉ inactive in causing reducing sugar release from barley seed halves. In their tests, seed halves were incubated 24 hr in 1 µg/ml hormone. In measurements of α-amylase induction, Jones and Varner (9) found that GA₁ and GA₃ were most active, GA₄ and GA₅ less active and GA₇ least active of the 5 compounds tested. The results of the work described here show a similar order of activity for these gibberellins in the induction of acid phosphatase.

![Fig. 2. Acid phosphatase release in response to seed half incubation temperature. Seed halves were incubated for 24 and 40 hr in 10⁻⁷ M GA₃. Water controls showed no phosphatase activity. Each value is the arithmetic mean of 5 replicates.](image1)

![Fig. 3. Time and rate of phosphatase release from seed halves in response to GA₃. Seed halves were incubated in 10⁻⁷ M GA₃ at 29°. Each value is the arithmetic mean of 5 determinations. Time-course of reducing sugar and α-amylase release, in arbitrary units, is included for comparison. The maximum value of α-amylase observed (72 hr) corresponded to a change in absorbancy of a starch-KI complex of 0.73 optical density units, or 66 µg α-amylase equivalents per seed half. The maximum value of reducing sugar (69 hr) corresponds to 6.2 mg maltose equivalents released per seed half.](image2)

![Fig. 4. Effect of GA₃ concentration on acid phosphatase from barley seed halves. Seed halves were incubated 44 hr at 29°. Each value is the arithmetic mean of 5 determinations.](image3)
In these tests, a considerably longer period was required for the onset of α-amylase activity than reported by Varner and Ram Chandra (15). These investigators found α-amylase activity in the incubation liquid after 9 hr compared to 26 hr observed in this study. Apparently the time difference is due to differences in technique rather than differences in seed. Varner and Ram Chandra imbibed Himalaya variety seed halves 3 days on moist sand and then incubated them in a buffered solution on a shaker. When the same technique is used with White Naked Atlas variety, α-amylase release begins 10 to 12 hr after the start of incubation (Corcoran, M. R., unpublished).

Reducing sugar and phosphatase measurements from identical incubation liquids show that the order of activity of the 12 compounds is exactly the same in both assays; relative activities in the 2 assays are similar, but not identical. The similarity in the ability of these compounds to induce reducing sugar and phosphatase release suggests 2 possible bases for gibberellin activity. A possible explanation is that activity is determined by the structure of the molecule and the permeability of the cell to it. Activity generally increases with oxidation level and specific relationships between structure and activity are generally similar to those reported from whole seedling assays (2). However, at least 3 effects of structural changes on biological activity in half seeds may be noted which differ from effects reported from seedling assays. First, hydration of the C-8 olefinic bond (Fig. 5) increases activity, since GA₂ is more active than GA₁; GA₁ is more active than GA₂ on seedlings. Second, the olefinic bond between C-3 and C-4 does not influence activity, since GA₁ is equally active as GA₃, and GA₄ is equally active as GA₅; GA₃ and GA₅ are generally more active than GA₁ and GA₄, respectively, in seedling assays. Third, carboxylation of the C-4a methyl increases activity in the barley half seed assay, since GA₁₃ is more active than GA₁₄; GA₁₃ is generally less active than GA₁₄ in seedling tests.

If the explanation is correct that activity is determined by the structure of a compound, then reducing sugar and phosphatase release must be induced by the same mechanism. It seems unlikely that 2 separate mechanisms would be so similar in response to each of the 12 compounds. A single mechanism was suggested by Paleg et al. (13) to explain similar relative activities in different tests. They found that 8 different gibberellins showed the same pattern of responses in inducing the release of reducing sugar and total protein.

An alternate explanation for similarity in activity in 2 test systems is that the compounds are converted to a single form which is responsible for both the reducing sugar and phosphatase responses. If a compound is metabolized to an active product, then its activity would depend on the efficiency of its conversion to the active form and similar relative activities would be expressed in different test systems. It is interesting to note that activity generally increases in the order in which compounds are produced in a pathway from kaurene to GA₉. Studies with G. fujikuroi show that kaurene is oxidized to C₂₀ gibberellins which in turn are converted to C₁₉ gibberellins (5, 6, 16). Eight of the compounds used here are produced in the order: kaurene, kaurenoic acid, GA₄₄, GA₃₃, GA₂₈ and GA₁₇, and GA₁ and GA₂. The ability of these compounds to induce phosphatase and reducing sugar release from barley seed halves increases in exactly the same order. Although GA₉ is produced from kaurene in G. fujikuroi, it is not a precursor to GA₉ (5). This could account for its relatively low activity when compared to GA₁₃ and GA₁₄. GA₂ is probably produced by hydration of GA₄ and its relatively high activity might reflect its proximity to GA₁ in the pathway. Pathways leading to GA₁₃ and GA₉ are unknown, so the activity of these compounds cannot be related to their biosynthesis.

Interpretation of relative activity data must also consider the possibility of active impurities in compounds being tested. Activities were determined from tests which spanned a thousand-fold range of concentrations. A highly active hormone contaminating a solution of hormone with low activity might be responsible for some or all of the activity assigned to the less active compound. This would be especially true of compounds whose separation is difficult because of similarity in structure.

The response of barley seed halves to low concentrations of gibberellins and gibberellin precursors

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### Table I. Relative Activities of 12 Gibberellins and Gibberellin Precursors in 2 Assays

<table>
<thead>
<tr>
<th>Conc.</th>
<th>Assay</th>
<th>Relative activity</th>
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<tbody>
<tr>
<td>10⁻⁸ M</td>
<td>Phosphatase</td>
<td>GA₅₁₀₀ = GA₄₁₀₀</td>
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<tr>
<td></td>
<td>Reducing sugar</td>
<td>GA₄₁₀₀ = GA₃₁₀₀</td>
</tr>
<tr>
<td>10⁻⁷ M</td>
<td>Phosphatase</td>
<td>GA₃₁₀₀ &gt; GA₂₈₇</td>
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<tr>
<td></td>
<td>Reducing sugar</td>
<td>GA₃₁₀₀ &gt; GA₄₇₁</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>Phosphatase</td>
<td>GA₁₃₁₀₀ = GA₃₁₀₀</td>
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<td></td>
<td>Reducing sugar</td>
<td>GA₁₃₁₀₀ = GA₄₅₀</td>
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<tr>
<td></td>
<td>Reducing sugar</td>
<td>GA₂₅₀ &gt; GA₁₃₃₄</td>
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shows that phosphatase release can be used for the bioassay of gibberellin-like substances. Acid phosphatase tests are at least as sensitive as those based on reducing sugar or amylase secretion (9) and the assay is convenient to perform and provides results relatively quickly.

One advantage stressed for the amylase assay over the reducing sugar assay is that amylase measurement is closer to the primary site of action of gibberellins and perhaps less susceptible to non-specific interferences (9). Phosphatase measurement offers the same advantages over both the reducing sugar and amylase assays, since phosphatase release occurs more quickly than reducing sugar or amylase release. One possible disadvantage is the presence of endogenous phosphatase in the seed half (11) which may be released into the incubation liquid spontaneously. However, when tests are carried out as described here, endogenous phosphatase is not released to a significant extent.

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Literature Cited


