Immunoaffinity Techniques Applied to the Purification of Gibberellins from Plant Extracts

Richard C. Durley*, C. Ray Sharp, Sonia L. Maki1, Mark L. Brenner, and Michael G. Carnes2

Monsanto Company, Saint Louis, Missouri 63198 (R.C.D., C.R.S., M.G.C.); and Department of Horticulture Science and Landscape Architecture, University of Minnesota, Saint Paul, Minnesota 55108 (S.L.M., M.L.B.)

ABSTRACT

The use of immunoaffinity columns containing anti-gibberellin (GA) antibodies for the selective purification of GAs in plant extracts is described. GA1, GA3, GA4, GA6, GA7, and GA8 conjugates to bovine serum albumin were synthesized and used to elicit anti-GA polyclonal antibodies (Abs) in rabbits. Protein A purified rabbit serum, containing a mixture of anti-GA Abs, was immobilized on matrices of Affi-gel 10 or Fast-Flow Sepharose 4B. Columns of these immunosorbents retained a wide range of C-19 GA methyl esters, but no C-20 GA methyl esters. Quantitative recovery of C-19 GA methyl esters was achieved from the columns, which, after reequilibration in buffer, could be reused up to 500 times. The immunosorbents were tested by examination of extracts from immature soybean and pea seeds. GAs were initially purified by passing the extracts through DEAE-cellulose and concentrating them on octadecylsila. The extracts were methylated and further purified on the mixed anti-GA immunoaffinity columns. GAs were detected and quantified as methyl esters or methyl ester trimethylsilyl ethers by gas chromatography-mass spectrometry-selected ion monitoring. GA1 was found in soybean seeds, 17 days after anthesis, at low levels (8.8 nanograms per gram fresh weight. C-19 GAs were examined in cotyledons, embryonic axes, and testae of G2 pea seeds harvested 20 days after anthesis. High levels of GA5 and GA2 were found in cotyledons (3580 and 310 nanograms per gram fresh weight, respectively) and embryonic axes (5375 and 1430 nanograms per gram fresh weight, respectively). Lower levels of GA2 were found in cotyledons and embryonic axes (147 and 161 nanograms per gram fresh weight, respectively). GA5 was the major GA of testae at levels of 185 nanograms per gram fresh weight. Trace quantities of GA10 and GA11 were also observed in testae.

Imunoaffinity purification is an alternative to immunological analysis, which has been successfully applied to the detection and quantitation of plant hormones (19). Instead of utilizing the antiphytohormone Ab by as a means of detection of hormones in a partially purified extract, the Ab is immobilized on a solid matrix and the resultant immunosorbent

1 Supported by a grant from the Herman-Frasch Foundation.
2 Current address: Ciba-Geigy Corp., P. O. Box 12257, Research Triangle Park, NC 27709-2257.
3 Abbreviations: Ab, antibody; amu, atomic mass unit; C18, octadecysila; GC-MS-SIM, gas chromatography-mass spectrometry-selected ion monitoring; IgG, immunoglobulin G; MeTMSI, methyl ester trimethylsilyl ether; PAH, p-aminohippuric acid.

used to selectively purify hormones in the extract. The phytohormones can be recovered from the immunosorbent in sufficiently pure form for direct analysis by high resolution techniques such as HPLC, GLC, or GC-MS. Immunoaffinity methodologies have been described for cytokinins (7, 8, 14, 16), abscisic acid (7), 3-indole acetic acid (17), and for GA3 (12).

A major challenge of GA analysis is the diverse structure of these phytohormones. It would be too tedious to examine plant extracts with immunosorbents which were specific for individual GAs (12). It is therefore desirable to prepare immunosorbents which bind as many GA structural types as possible. In this report we describe the preparation and use of mixed anti-GA immunoaffinity columns which retain a wide range of C-19 GAs, and the use of these columns in the examination of GAs in soybean and pea seeds. A preliminary report of this work, together with similar methodology for other phytohormones, has been presented (8).

MATERIALS AND METHODS

Labeled GAs

[3H]GA1 (32 Ci/mmol) and [3H]GA4 (38 Ci/mmol) were purchased from Amersham Corporation. [3H]GA3 (2.3 Ci/mmol) was synthesized from GA3 (15). [3H]GA28 (28 Ci/mmol) was a gift from A. Crozier (Glasgow University).

Preparation of GA-Protein Conjugates

Prior to conjugation of gibberellins, a PAH linker was attached to BSA (18). BSA (1.5 g) and PAH (1.5 g) were dissolved in water (500 mL) and the solution was adjusted to pH 8.0 with 2 N NaOH. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (1.5 g) was added and the solution stirred at room temperature and adjusted to pH 6.4 with 0.2 N HCl. The stirring was continued for 4 h, during which time the pH was occasionally adjusted to 6.4. At this time precipitation started to occur indicating that sufficient substitution on BSA had occurred. The solution was dialyzed against water for 3 d, then freeze-dried. The substitution of PAH in the PAH-BSA was calculated by measurement of the absorption at 280 nm and found to be 18 mol of PAH per mol of BSA.

GA1, GA3, GA4, GA6, GA7, and GA8 were each conjugated separately to PAH-BSA via conversion to their anhydrides (2). Each GA (25 mg) and dicyclohexyl carbodiimide (7.5 mg) were stirred in dry dioxane (0.45 mL) in a sealed Reacti-Vial (Pierce Chemical Co.) for 16 h. After centrifugation to remove
the precipitated dicyclohexylurea, the clear solutions were added to a stirred solution of PAH-BSA (18 mg) in ice-cold dimethylformamide (0.25 mL) and water (0.25 mL) over a period of 1 h. The solutions were stirred at 4°C overnight. The solutions were dialyzed against PBS (0.01 M K2HPO4, 0.15 M NaCl [pH 7.4]) for 24 h and water for 48 h. Dialysis solutions were changed periodically. The purified solutions containing the conjugates were freeze-dried and the lyophilized proteins stored at -20°C. The molar coupling ratios of GA1, GA4, GA5, and GA6 to PAH-BSA was determined by the addition of 80,000 dpm of the respective 3H-labeled GA prior to conversion to anhydrides and determining the radioactivity in an aliquot from the conjugates. These values were found to be 10, 18, 16, and 19 mol of GA per mol of protein, respectively.

Immunization Protocol

Approximately 200 μg of each of the six GA PAH-BSA conjugates were dissolved in PBS and emulsified with Freund's complete adjuvant. The mixture containing all six conjugates was introduced by subcutaneous and intramuscular injection into rabbits. Rabbits were boosted every 3 weeks with 1.2 mg of antigens in PBS and Freund's incomplete adjuvant. After three such injections, rabbit sera were tested by serial dilution with the methyl esters of [3H]GA1, [3H]GA4, [3H]GA5, and [3H]GA6 for Ab titer. Rabbits showing measurable serum titer to both esters were further boosted with 200 μg (total) of mixed conjugates, and this was repeated every 3 weeks. When titers to above labeled GAs reached 2,000 to 3,000, rabbits were bled. Blood was collected from ear veins 12 d after each 200 μg boost, then clotted and centrifuged. Serum was stored at -20°C.

Immunosorbent Preparation

IgG was purified from other sera proteins by passing through 15 mm × 25 cm columns of protein A-Sepharose (Sigma Chemical Co.). After washing with 10 column volumes of PBS, the IgG was eluted with 0.5% acetic acid/0.15 M NaCl solution. The IgG was dialyzed against PBS and concentrated by vacuum dialysis. The concentration of IgG in PBS was calculated from absorption at 280 nm.

Purified IgG was attached to Affi-Gel 10 support (Bio-Rad) by the following procedure. IgG in PBS was added to a slurry of Affi-Gel 10 support in coupling buffer (0.1 M NaHCO3 [pH 8.0]). Typical quantities were 20 to 40 mg of IgG per mL of gel support. The slurry was tumbled for 4 h at 4°C. The immunosorbent was centrifuged and the supernatant removed to measure unbound protein at 280 nm for determination of coupling efficiency, which was found to be 93% of the IgG added. The gel was washed with PBS and resuspended in 1.0 M ethanolamine (pH 8.0) for 2 h at room temperature. The filtered immunosorbent was washed with PBS and poured into small polypropylene columns (Isolab Inc., Akron, OH) in volumes of 1 to 2 mL.

Alternatively, purified IgG was attached to Fast Flow Sepharose CL-4B support (Pharmacia) via carbonyldimimidazole activation (3). Fast Flow Sepharose CL-4B (5 mL) was washed on a sintered funnel with water (10 mL), acetone/water (1/1, 10 mL), and acetone (3 × 5 mL). The gel was added to 1,1-carbonyl-diimidazole (300 mg in 6 mL acetone) and tumbled at room temperature for 15 min. The gel was filtered and washed with acetone followed by coupling buffer (0.05 M Na2HPO4/0.1 M NaCl [pH 7.0]). The gel then was suspended in coupling buffer containing 65 mg of IgG and the mixture tumbled at 4°C for 20 h. The mixture was centrifuged and the supernatant removed to measure unbound protein at 280 nm to determine coupling efficiency, which was found to be 90% of IgG added. The gel was resuspended in 1.0 M ethanolamine at pH 8 and the mixture tumbled for 12 h at 4°C. The filtered immunosorbent was washed with PBS and poured into small polypropylene columns in volumes of 1 to 2 mL.

Immunosorbent Treatment and Storage

Columns were washed until the absorption at 280 nm was equal to background absorption. The columns were stored at 4°C in PBS containing 0.01% sodium azide. Prior to use, methanol was passed through the columns to remove any methanol soluble impurities. The columns were then immediately reequilibrated in PBS.

Immunosorbent Characterization

The capacities of the Affi-Gel 10 and Fast Flow Sepharose immunosorbents, at two IgG loadings, were determined for GA1, GA4, and GA6. Samples of each GA ranging from 100 to 2,000 ng were prepared and to each sample was added an internal standard of the respective 3H-labeled GA (50,000 dpm). These were dissolved in methanol and treated with excess diazomethane in ether. The recovered methyl esters were added to immunoaffinity columns (3 mL) in PBS (5 mL) at room temperature, and the columns were washed with PBS (5 mL) and water (5 mL). Flow rates for loadings and washings were 1 to 2 mL/min. Retained GA methyl esters were eluted with methanol (7 mL), and the columns were immediately reequilibrated with PBS. The tritium in the methanol washes was counted by liquid scintillation to determine amount of GA methyl ester retained. These values were graphed against applied GA methyl ester. Extrapolations from the graph enabled the saturation column capacity for each GA methyl ester to be calculated.

The range of absorptive capacity was determined by using the Affi-Gel 10 immunosorbent (35 mg IgG/mL gel) and 16 GA methyl esters, spiked in an extract of soybean leaves. Frozen soybean leaves (1 g) were homogenized in methanol/water (4:1, 5 mL) at 4°C, and the extracted material was purified through a DEAE column and concentrated on a C18 column (for details, see below). The purified extract was spiked with GA1, GA3, GA4, GA5, GA7, GA9, GA13, GA14, GA18, GA20, GA23, GA27, GA29, GA34, GA36, and GA37 (20–40 ng of each) and the mixture was methylated with diazomethane. The methyl esters were dissolved in methanol (0.2 mL) and diluted to 8 mL with PBS. The solution was pass at room temperature through an anti-GA Affi-Gel 10 immunosorbent (35 mg IgG/mL gel, 3 mL), which was washed with water (10 mL). GA methyl esters were recovered in methanol, derivatized, and examined by GC-MS-SIM (for details, see
below). Recoveries of each GA were estimated by comparison of TIC or individual ion GAME-TMSi peak areas with those of a standard GAME-TMSi mixture.

Plant Material

Soybeans (Glycine Max L., cv Wayne) were greenhouse grown with 30°C day/19°C night temperatures and a supplemental photoperiod of 16 h. Peas (Pisum sativum of the genetic line G2) were grown in a growth chamber with 19°C day/17°C night under a photoperiod of 18 h. Two plants were grown in 4 L pots in 1:1 sand:soil:peat. They were fertilized weekly with commercial fertilizer (Peters, Allentown, PA). Seeds (2 mm diameter) were harvested 17 d (soybean) and 20 d (peas) after anthesis. Soybean seeds were not dissected, but pea seeds were dissected into cotyledons, embryonic axes, and testae. The average fresh weight of a whole pea seed before dissection was 275 mg.

Extraction and Purification of Plant Material

Frozen seeds, seed parts, or leaves (0.07–3 g) were homogenized in methanol (1–5 mL, depending on weight of tissue) containing sodium diethyldithiocarbamate (50 mg) and butylated hydroxytoluene (5 mg). [3H]JA1 (106 dpm) was added as internal standard. After centrifugation (20,000g, 15 min, 4°C), the supernatant was concentrated to half-volume and then diluted seven times with ammonium acetate buffer (20 mM [pH 6.5]). This solution was passed through a DEAE-cellulose column (DE-52, Whatman, 7–20 mL), which was washed with 2 column volumes of ammonium acetate buffer (20 mM [pH 6.5]). The GAs were eluted with 2 column volumes of 1 M acetic acid (adjusted to pH 3.1 with aqueous ammonia) directly onto a C18 column (Seprlyte, Analyti-chem, 1.5–2.0 mL). The C18 column was washed with water (7 column volumes), and the GAs were eluted with methanol (3 column volumes), which was taken to dryness in vacuo.

The methanol extract was taken up in methanol (0.2 mL), diluted with PBS (8 mL), and passed through the Affi-Gel 10 immunosorbent (35 mg IgG/mL gel, 3 mL column) at room temperature and at flow rates of 1–2 mL/min. The column was washed with PBS (10 mL) and water (5 mL). GA methyl esters were recovered in methanol (7 mL) and a sample was removed for counting to determine purification efficiency. Typically, the purification efficiency was around 60%. The remainder of the solution was taken to dryness in vacuo. Purified samples were stored at −70°C.

GC-MS-SIM

Samples of GA methyl esters, or immunoaffinity-purified extracts, were transferred to 0.3 mL conical Reacti-Vials (Pierce Chemical Co.), evaporated under nitrogen, and dried in vacuo over KOH. BSTFA + 1% TMCS (Pierce Chemical Co.) (10–20 µL) was added. The solutions were sealed, mixed, and left overnight at room temperature in a desiccator over KOH. The samples (5 µL) were injected via a heated injector into the GC-MS-SIM with splitless flow. A Hewlett-Packard GLC model 5890 was connected to a Hewlett-Packard MSD model 5990 with data processing by a Hewlett-Packard 59970B work station. The DB-5 fused silica column (0.2 mm ID × 30 m) had film thickness 0.25 microns (J and W Scientific) and helium was the carrier gas. The column temperature was initially at 140°C for 6 min, then ramped at 15°C/min to 220°C, held for 8 min, then ramped at 1°C/min to 260°C.

Monitored ions were changed in six time sequences according to retention times of the derivatized GAs. The time sequences, corresponding GAs (as MeTMSi derivatives, except for GAs, which was as a methyl ester only) and monitored ion were: 15–29 min, GAs: m/z 330, 298, 270, 243, 226, 227, 217; 29–35.2 min, GAs, GA4, GA7: m/z 418, 416, 403, 401, 390, 388, 375, 359, 357, 301, 299, 298, 287, 208, 207; 35.2–38 min, GAs, GA5: m/z 418, 416, 384, 355, 328, 289, 284, 225, 224, 223, 222; 38–42.5 min, GA13, GA18, GA34: m/z 536, 506, 477, 436, 400, 390, 319, 310, 241, 223, 217, 208, 207, 129; 42.5–48 min, GA1, GA3, GA23, GA29, GA36: m/z 550, 522, 506, 504, 491, 463, 448, 447, 375, 373, 370, 347, 303, 291, 223, 218, 217, 208, 207, 147; 48–60 min, GA27, GA57: m/z 594, 520, 448, 376, 375, 374, 223, 218, 217. In addition, for pea seeds, ions of GAs, MeTMSi, m/z 386, 284, 241, 225, 224 were monitored in time sequence 29–31.2 min.

GATMSIs were identified and quantified by comparison of individual ion traces with those of standards, or known spectra. Values were adjusted for purification efficiency of the [3H]GA1 standard. Sillation efficiency was not determined individually, although over 95% was obtained with standards of the same GAs identified. The standard GA derivatives were prepared and chromatographed at similar concentrations to extracted GAs.

RESULTS AND DISCUSSION

Eliciting Antibody Response

Due to the diversity of GA structure, the effectiveness of an immunoaffinity technique for GAs depends on the ability of the immobilized Abs to bind a range of structural types. In order to obtain such Abs, protein conjugates of GA1, GA3, GA4, GA5, GA7, and GAs were constructed. These represented various common structural features of the GAs, including ring A and rings C/D hydroxylation, and saturation/unsaturation in ring A. C-20 GAs were not examined at this time. Since Abs against individual GAs were not required, all six protein conjugates were mixed in approximately the same amounts and the mixture used to elicit anti-GA Abs in rabbits. Titters were determined by [3H]GA1 and [3H]GA5. Only rabbits showing titer to both these structurally diverse GAs were boosted and bled for anti-GA Abs.

Preparation of Immunoabsorbents

IgG was purified from other sera proteins by passing rabbit sera showing GA1 and GA5 titer through Protein A-Sepharose. The purified IgG, after dialysis and concentration, was bound directly to sorbents without further purification. Anti-GA IgG was bound to sorbents Affi-Gel 10 and Fast Flow Sepharose.
CL-4B. Although both sorbents bound IgG well, a higher binding to Affi-Gel 10 could be obtained (Table I). The GAs were converted to methyl esters and these were applied in PBS or other buffers to small 3 mL columns of the immunosorbents. The columns were washed with buffer and water, and the GA methyl esters eluted with methanol. Various chaotropic reagents and changes in pH were examined, but none were effective as methanol. Furthermore, the GA methyl esters could be recovered from methanol by simple evaporation in vacuo. Only GA methyl esters bound to the immunosorbents. No binding of free GA acids was observed (data not presented) (cf. ref. 2).

The maximal column capacities for GA, GA, and GA methyl esters were determined for each immunosorbent at two protein loadings (Table I). The capacities varied with the GA, from GA, methyl ester (lowest) to GA, methyl ester (highest), and also with protein loading. Affi-Gel 10, with a maximal protein loading of 35 mg/mL gel, exhibited the highest capacity. In contrast to proteins, which can be difficult to remove from immunosorbents at high IgG loadings, due to multiple site attachment, small molecules such as GA derivatives are released with ease. Hence, for small molecules, high IgG loading is advantageous to maximize column capacity, limit immunosorbent required, maximize flow rates, and minimize methanol volume needed to remove phytohormones.

In order to obtain information concerning the range of GA structural types that can be bound to the mixed anti-GA immunosorbent, recoveries of 16 GA methyl esters were determined from Affi-Gel 10 with 35 mg IgG/mL gel in the presence of a plant extract (soybean leaves) which contained no detectable GAs (<0.1 ng). The amount 20–40 ng of each GA methyl ester passed through the column was much lower than the estimated capacity of the column for that type of GA derivative (Table I). GA methyl ester recovery was estimated by conversion to the GAmETMSi derivatives and examination by GC-MS-SIM (Fig. 1). All C-19 GA methyl esters were recovered in over 90% yield (Table II). These included GA methyl esters which were hydroxylated at either C-1, C-2, C-3, and C-13 or nonhydroxylated, and GA methyl esters which were saturated or unsaturated in ring A. The extract did not interfere with GA binding or GC-MS-SIM analysis. C-20 GA methyl esters were not retained by the immunosorbent. Hence the Abs, generated against C-19 GA-BSA conjugates, did not recognize GA methyl esters with a C-20 carbon function.

Immunaffinity columns can be reused multiple times. Some columns were used up to 500 times before discarding. It is important that column capacity far exceed the phytohormone content of the sample (10- to 100-fold). This ensures complete phytohormone recovery, even in the presence of competing phytohormones or interfering substances in the extract.

**Extract Purification**

Although active sites on the immunosorbent were blocked with ethanolamine, other factors such as the sorbent itself, the linkage, and the IgG protein can cause nonspecific binding. Therefore, unless the extract contained few impurities (rare for plants) an initial anion exchange column was found to be necessary to remove phenolics and other substances of high binding characteristics. If this initial purification were excluded, sample dry weights were too large for derivatization in minimal solvent volume for GC-MS-SIM analysis and the chromatograms obtained were difficult to interpret (data not given). The method involving absorption on DEAE-cellulose followed by concentration on C18 was found to be ideal for the initial purification. After recovery and conversion to methyl esters, the extracted phytohormones were sufficiently pure to be applied to the immunosorbent column.

Since the protocol utilizes only low pressure apparatus, multiple samples may be purified simultaneously. Furthermore, since protocols overlap, it is possible to use this methodology in a comprehensive scheme that also isolates other classes of hormones (7, 8).

**Detection and Quantitation**

GC-MS-SIM was employed for detection and quantitation of the GAs as MeTMSi derivatives. Groups of ions were examined in specific elution time sequences. During each time sequence ions were examined which were abundant and characteristic of the GAmETMSi derivatives eluting in that time sequence (Fig. 1). Detection limits were in the 1 to 2 ng range. The examination of characteristic ions provided further selectivity which complimented the immunaffinity purification. Nonspecific binding of potentially interfering substances in the extract to the immunosorbent was minimal.

---

**Table I. Anti-GA Immunosorbent Capacities**

Values derived by extrapolations from graphs of [3H]GA methyl ester applied to a 3 mL column of anti-GA immunosorbent versus [3H]GA methyl ester retained.

<table>
<thead>
<tr>
<th>Immunosorbent</th>
<th>IgG Loading</th>
<th>[3H]GA Methyl Ester Retained at Saturation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/mL gel</td>
<td>GA&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>Affi-Gel 10</td>
<td>20</td>
<td>300</td>
</tr>
<tr>
<td>Affi-Gel 10</td>
<td>35</td>
<td>390</td>
</tr>
<tr>
<td>Fast-Flow Sepharose CL-4B</td>
<td>8</td>
<td>140</td>
</tr>
<tr>
<td>Fast-Flow Sepharose CL-4B</td>
<td>14</td>
<td>190</td>
</tr>
</tbody>
</table>

---
were ion monitoring, characteristic ions or with the seeds. (Fig. 1). 

Examination of GAs in Soybean and Pea Seeds

The anion exchange/immunoaffinity/GCMS-SIM protocol was tested with extracts from immature soybean and G2 pea seeds. The tissues extracted, together with amounts, column sizes, and the GAs found are summarized in Table III.

The extracted and purified GAs, as MeTMSi derivatives, were identified and quantified by examination of individual characteristic ions in the TIC traces (Figs. 2–6) and comparison with ions in TIC traces of standard samples, if available, or comparison with published spectra (5). TIC traces of the purified GAMeTMSi derivatives, with sequential changes in ion monitoring, were free of major impurity peaks (Fig. 2), making interpretation simple. The recovery efficiency was estimated from determination of an internal reference standard of high specific activity [3H]GA1 and was generally found to be approximately 60%. The reference was counted just prior to trimethylsilation. The efficiency of silation was not determined for individual extracts, but was typically >95% when spiked extracts were compared to standards.

GA1 was clearly identified in soybean seed (Fig. 3), confirming an earlier report (4). No other gibberellins could be definitively identified. Observation of ions suggested that GA4 may be present in trace amounts (data not presented). The seed concentration of GA27 was low (Table III), several orders of magnitude lower than the levels of GAs normally found in other immature legume seeds, e.g. beans (9) or peas (11).

GAs, GA20, GA29, and GA31 were detected in immature G2 pea seeds. The first three of these GAs have previously been found in this genetic line of pea seeds (6). GA20 was found in cotyledons (Fig. 4) at high levels, but even higher levels were found in embryonic axes (Table III). High levels of GA29 were also found in embryonic tissue (Fig. 5; and Table III). In contrast, testae largely contained GA3 (Fig. 6; Table III), together with trace levels of GA30 and a GA3 metabolite, GA51, GA51 was identified by comparison of the major ions, m/z 386 (abundance 17%), 284 (100%), 241 (39%), 225 (82%), 224 (34%), in a peak corresponding to the expected retention time of GA51,MeTMSi to the major ions, m/z 386 (abundance 25%), 284 (100%), 241 (24%), 225 (91%), 224 (42%), in the mass spectrum of GA51,MeTMSi. GA20-catabolite (10), a nonlactonic, α,β-unsaturated ketone, was not observed, indicating that, if it is present in immature G2 pea seeds, the 4 → 10 lactone moiety is necessary for Ab recognition.

Advantages of the Immunoaffinity Technique

The immunoaffinity method for GAs described herein selectively purified these phytohormones from relatively crude plant extracts. This is in contrast to more traditional chromatographic methods which are nonselective and often have the disadvantage of separating phytohormones into multiple fractions during purification. By employing C-19 GAs of specific structural types to eliciting antibodies, mixed anti-GA immunosorbents were prepared that bound a wide range of C-19 GAs. This method is therefore more universal than

---

**Table II. Recoveries of 16 GA Methyl Esters from Anti-GA Affi-Gel 10 Immunosorbent**

A methylated mixture of 16 GAs (20–40 ng each) and a DEAE/C18-purified soybean leaf extract, which by itself contained no detectable GAs, was applied to a 3 mL anti-GA immunosorbent column. The recovered GAs methyl esters were converted to MeTMSi derivatives and these were examined by GC-MS-SIM. Recoveries were estimated by comparing the TIC or individual ion peak areas for each GAMeTMSi with those obtained from reference samples.

<table>
<thead>
<tr>
<th>GA Structural type</th>
<th>GAMeTMSi</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-19</td>
<td>GA1</td>
<td>95.9</td>
</tr>
<tr>
<td></td>
<td>GA3</td>
<td>94.0</td>
</tr>
<tr>
<td></td>
<td>GA4</td>
<td>97.5</td>
</tr>
<tr>
<td></td>
<td>GA5</td>
<td>97.7</td>
</tr>
<tr>
<td></td>
<td>GA7</td>
<td>98.1</td>
</tr>
<tr>
<td></td>
<td>GA9</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td>GA20</td>
<td>97.2</td>
</tr>
<tr>
<td></td>
<td>GA29</td>
<td>91.6</td>
</tr>
<tr>
<td></td>
<td>GA24</td>
<td>97.9</td>
</tr>
<tr>
<td></td>
<td>GA34</td>
<td>90.6</td>
</tr>
<tr>
<td></td>
<td>GA37</td>
<td>90.1</td>
</tr>
<tr>
<td>C-20</td>
<td>GA13</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>GA14</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>GA18</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>GA23</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>GA27</td>
<td>0.0</td>
</tr>
</tbody>
</table>

(Fig. 1). No major peaks that could be confused with GAs were observed in the absence of GAs.
Table III. GA Extraction Details and Tissue Concentrations in Immature Whole Soybean Seeds and Pea Seed Parts

Soybean and G2 pea seeds were harvested 17 and 20 d after anthesis, respectively. GA tissue concentrations were estimated as in Table II and are means of three replicates ± se. Values were corrected for purification efficiency with [3H]GA, as internal standard. Sialation derivatization efficiency was not determined individually, although over 95% was obtained with standards of the identified compounds prepared at same concentration.

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Extracted Amount</th>
<th>Column Sizes</th>
<th>GA Found</th>
<th>Tissue Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DE52</td>
<td>C18</td>
<td>Immunoaffinity</td>
<td></td>
</tr>
<tr>
<td>Whole Soybean Seeds</td>
<td>5.00</td>
<td>20</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>G2 pea cotyledons</td>
<td>0.15</td>
<td>7</td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2 pea embryonic axes</td>
<td>0.07</td>
<td>7</td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2 pea testae</td>
<td>2.00</td>
<td>15</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. The TIC trace of GC-MS-SIM of MeTMSi derivatives of a sample extracted from pea seed embryos and purified by the DEAE/C18 and mixed anti-GA immunoaffinity protocol. See Figure 1 and "Materials and Methods" for sample treatment and GC-MS-SIM conditions.

Figure 3. GC-MS-SIM of GA7MeTMSi extracted from immature soybean seed after purification by the DEAE/C18 mixed anti-GA immunoaffinity protocol. See Figure 1 and "Materials and Methods" for sample treatment and GC-MS-SIM conditions. Major ions and ion intensities of a GA7MeTMSi reference sample: m/z 416 (15), 384 (43), 356 (61), 281 (28), 223 (92), and 222 (100).

Figure 4. GC-MS-SIM of GA20MeTMSi extracted from immature G2 pea seed cotyledons after purification by the DEAE/C18 mixed anti-GA immunoaffinity protocol. See Figure 1 and "Materials and Methods" for sample treatment and GC-MS-SIM conditions. Major ions and ion intensities of a GA20MeTMSi reference sample: m/z 418 (100), 403 (17), 390 (8), 375 (48), 359 (12), and 301 (18).
Figure 5. GC-MS-SIM of GA$_29$MeTMSi extracted from immature G2 pea seed embryos after purification by the DEAE/C18 mixed anti-GA immunoaffinity protocol. See Figure 1 and “Materials and Methods” for sample treatment and GC-MS-SIM conditions. Major ions and ion intensities of a GA$_29$MeTMSi reference sample: m/z 506 (100), 491 (10), 447 (7), 375 (14), 303 (21), 291 (8), and 207 (42).

Figure 6. GC-MS-SIM of GA$_4$MeTMSi extracted from immature G2 pea seed testae after purification by the DEAE/C18 mixed anti-GA immunoaffinity protocol. See Figure 1 and “Materials and Methods” for sample treatment and GC-MS-SIM conditions. Major ions and ion intensities of a GA$_4$MeTMSi reference sample: m/z 330 (9), 298 (100), 270 (71), 243 (44), 227 (39), 226 (38), and 217 (28).

current GA radioimmunoassays or enzyme-linked immunoassays, which tend to be structure specific and to exhibit limited cross-reactivity between GAs (1, 2, 13, 20).

ACKNOWLEDGMENT

The authors gratefully acknowledge the gift of $^3$HGA$_4$ from A. Crozier (Glasgow University).

LITERATURE CITED


