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GIBBERELLIN A_{12} AND GIBBERELLIN A_{12}-7-ALDEHYDE AS ENDOGENOUS COMPOUNDS IN DEVELOPING SEEDS OF *PISUM SATIVUM*

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Pisum sativum*; Leguminosae; gibberellin A_{12}; gibberellin A_{12}-aldehyde; gas chromatography-mass spectrometry; isotope ratios; seed; pea.

Abstract—While gibberellin (GA) A_{12} and GA_{12}-7-aldehyde have been well established as intermediates in GA biosynthesis, their presence in higher plants has not been conclusively demonstrated, with the exception of GA_{12}-aldehyde in mature pea seeds. High specific activity [^{14}C] GA_{12} and [^{14}C]GA_{12}-aldehyde were synthesized enzymatically from [4, 5-^{14}C]mevalonic acid, using a pumpkin endosperm preparation, and purified by HPLC. These were added to developing pea seeds during homogenization and the radioactive material purified by HPLC. Mass spectra of GA_{12} and GA_{12}-aldehyde were obtained following gas chromatography. The ratio of the ^{12}C to ^{14}C mass ions for the radio-labelled GAs, and the seed extract with added radio-labelled GAs as internal standards, was examined. There was a 123 and a 579% enrichment of the ^{12}C present in the GA_{12} and GA_{12}-aldehyde spectra, respectively, relative to ^{14}C, proving the presence of endogenous GA_{12} and GA_{12}-aldehyde in the developing pea seeds. The amounts present were very low, namely 1.2 ng/g fresh weight for GA_{12} and 8.4 ng/g fresh weight for GA_{12}-aldehyde.

INTRODUCTION

Giberellin A_{12} (GA_{12}) was intially discovered in the fungus *Gibberella fujikuroi* [1]. Numerous studies on the biosynthesis of gibberellins (GAs) have indicated that GA_{12}-7-aldehyde is the first gibberellin in the pathway and that this is then converted to GA_{12} [2, 3]. The identification of both GA_{12} and GA_{12}-aldehyde has come mainly from analysis of intermediates of GA production in cell free systems following feeding of various precursors [3]. Actual identification of these compounds as endogenous compounds, particularly in higher plants, is rare despite their widely accepted presence on theoretical grounds as intermediates in the production of the GAs which accumulate at detectable levels. The reason for this is probably that the levels are very low because the metabolism of the compounds on to more persistent GAs is very rapid. GA_{12} has been listed as being present in apple seed as detected by GC/MS [4] but no confirming data was given. It has also been cited as having been detected, but as 'unpublished', in *Curcurbita maxima* seeds [5] and *Oryza sativa* [6]. GA_{12}-aldehyde has only been identified in mature dry pea seeds [7]. As part of our investigation of GA content and metabolism in peas we have succeeded in conclusively showing the presence of both these GAs as native compounds in developing pea seeds.

RESULTS AND DISCUSSION

In order to conclusively detect compounds present in extremely low amounts a method of purification with minimal loss has to be utilized. This is even more difficult with GAs as they possess no easily detectable physicochemical characteristic such as UV adsorption. Bioassay, which is commonly used for GAs, is also useless as the amounts present are too small, and both GA_{12} and GA_{12}-aldehyde are virtually inactive in most bioassays [8]. The approach we have taken is to add high specific activity [^{14}C]GA_{12} and [^{14}C]GA_{12}-aldehyde to pea seed extracts and to follow the radio-label through the purification of the extracts [9]. The principal GAs are then subjected to GC/MS analysis and the spectra of GA_{12} and GA_{12}-aldehyde detected. However, as even the most radioactive [^{14}C] compound is never 100% ^{14}C in the labelled position, but is a mixture of ^{12}C and ^{14}C species, account has to be taken of the contribution of the [^{12}C] compound added together with the ^{14}C tracer. We have done this by comparing the isotope ratios of the added tracer and the resultant tracer/endogenous GA mass spectra. Heavy isotope (e.g. ^{2}H^{13}C or ^{15}N) standards are usually used for the detection and quantitation of plant hormones using GC/MS [10] because of the high isotopic purity obtainable (usually <99%). However, due to lack of radioactivity, this method would not have succeeded in the present work because the radioactive internal standard is essential for monitoring the purification process. Thus even if the isotopic abundance of the standard is less than desirable it is still the method of choice for this work.

Gibberellin in A_{12} and GA_{12}-aldehyde were synthesized from high specific activity [4-^{14}C, 5-^{14}C]mevalonic acid using a cell-free preparation from pumpkin (*Cucurbita maxima*) endosperm. These GAs were then purified by HPLC. Mass spectra of the GA_{12} and GA_{12}-aldehyde showed that they contained 70.4 and 68.5% ^{14}C, giving

specific activities of 351.4 and 342.0 mCi/mmol, respectively (Table 1). These [^{14}C]GAs (1 μCi of each) were added to 200 g of pea seed following homogenizing and the GAs purified as described below. Through each HPLC step the ^{14}C was detected by an on-line radiomonitor that triggered a fraction collector to collect the radiolabelled material.

Separation of ^{2}H from ^{1}H containing compounds during HPLC has been noted [11]. Should such separation occur in our case, enrichment in ^{14}C would be expected, as all fractionation was made by following the radioactivity, yet enrichment was found in ^{12}C rather than ^{14}C. Also, as the separation of [^{12}C] and [^{13}C]compounds is minimal [12] it is likely that there would be minimal separation of the [^{14}C] and [^{12}C]GAs.

The purified GAs were methylated and repurified by HPLC prior to GC/MS. GC/MS was run in the scan mode searching for the spectra of the [^{12}C] and [^{14}C] GA_{12} and GA_{12}-aldehyde. The higher mass spectral lines of the [^{14}C]compounds are 16 mass units higher because eight ^{14}C atoms are incorporated into each GA. The presence of endogenous material is clearly seen as the proportion of ^{12}C changes from 42 and 46% to 94 and 312.5% of that of ^{14}C in GA_{12} and GA_{12}-aldehyde, respectively (Table 1). This represents a 123 and 579% enrichment, of ^{12}C present in the GA_{12} and GA_{12}-aldehyde respectively, relative to ^{14}C, caused by the addition of plant material. This is therefore conclusive proof of the existence of endogenous GA_{12} and GA_{12}-aldehyde in pea seed.

As the amount of [^{14}C]GA_{12} and GA_{12}-aldehyde added and the final isotope ratio is known, the amount of endogenous GA_{12} and GA_{12}-aldehyde in the pea seeds can be calculated as follows:

$$\text{Endogenous amount} = \frac{\text{Endogenous ion intensity}}{\text{Internal standard ion intensity}} \times \text{Amount of added internal standard}$$

$$\frac{\text{Total } ^{12}\text{C} - \text{internal standard } ^{12}\text{C ion intensities}}{\text{Internal standard } ^{12}\text{C} + \text{internal standard } ^{14}\text{C ion intensities}} \times \text{Amount of added internal standard.}$$

Using the ratios of the major ions for ^{12}C/^{14}C, i.e. *m/z* 300/316 and 241/257 for GA_{12} and GA_{12}-aldehyde, respectively.

The amounts are very low, as expected, with values of 1.2 ng/g fresh weight for GA_{12} and 8.4 ng/g fresh weight for GA_{12}-aldehyde. This contrasts with a level of 9.5 μg/g fresh weight of GA_{20} which has been recorded in pea seed at the same developmental stage [13]. In mature dry pea seed GA_{12}-aldehyde is present at the level of 17 ng/g fresh weight [7] which is similar to that reported in the present work when the decrease in water content of the seeds at maturation is taken into account. The GA_{12}-aldehyde in mature seeds is utilized during germination almost disappearing over the first 26 to 30 hr of germination [7]. The detection of GA_{12}-aldehyde in immature seeds indicates that it does not build up only during maturation of the seed, but is also present at earlier times of development. The level of endogenous GA_{12}-aldehyde is seven times that of GA_{12}. This is in agreement with the results of our [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} feeding studies with peas: [^{14}C]GA_{12} is metabolized several times more rapidly than [^{14}C]GA_{12}-aldehyde (J. W. Lee, P. J. Davies and A. Halinska, unpublished results) so that a lower endogenous content might be expected where GA_{12}-aldehyde is the metabolic precursor of GA_{12}.

EXPERIMENTAL

Plant material. Pea (*Pisum sativum* L.) plants of genetic line G2 [14] were grown in a peat–vermiculite mixture in 4l plastic pots, 2 plants per pot. The seed was sown in the greenhouse and the plants transferred 1 month later to growth chambers with photoperiods of 9 hr and 20° day and 17° night temp.

[^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} synthesis and purification. All glassware used was silanized with Aquasil. [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} were synthesized from [4-^{14}C, 5-^{14}C] mevalonic acid (MVA) using a cell-free system from the liquid endosperm of *Cucurbita maxima* Duchesne, cv. Big Max. Pum-

Table 1. Relative intensities of the high M_r mass ions in the mass spectra of the methyl esters of aldehyde recovered from pea seeds including the added

		m/z (%)				
[^{14}C]GA_{12} added	^{12}C	328(11)	301*(26)	300(42)	285(15)	241*(27)
	^{14}C	344(25)	317(14)	316(100)	301*(26)	257(23)
GA_{12} from peaseed	^{12}C	328(25)	301*(40)	300(94)	285(23)	241*(37)
	^{14}C	344(25)	317(12)	316(100)	301*(40)	257(22)
[^{14}C]GA_{12}-aldehyde added	^{12}C	330(6)	287(16)	270(21)	255*(53)	242(17)
	^{14}C	346(14)	303(14)	286(48)	271(20)	258(14)
GA_{12}-aldehyde from pea seed	^{12}C	330(22)	287(23)	270(59)	255*(32)	242(27)
	^{14}C	346(10)	303(5)	286(21)	271(26)	258(4)

The relative intensity of each ion, as per cent of the major ion, is given in parentheses after the ion ratio of the major ion for ^{12}C and ^{14}C.

*Mass ion occurs in both ^{12}C and ^{14}C spectra.

pkin fruits with the correct colour were sampled *in situ* to estimate the stage of seed development, and were harvested at 20% of the maximum cotyledon length, the stage reported to have the most activity [15]. Cell-free extracts and reactions with MVA were prepared essentially as ref. [15]. Extraction was done with 1 vol. of buffer A (40 mM HEPES, 0.6 M mannitol, 2.5 mM $MgSO_4$, 1 mM dithiothreitol, and 1 g/l BSA adjusted to pH 8.0 with KOH) per vol. of endosperm. Dialysis was 3 × against about 25 vol. of buffer A without BSA for 1, 0.75 and 0.75 hr. The volume of endosperm recovered was 104 ml to give a total enzyme extract of 280 ml. Per ml of reaction mixture, 0.65 ml of enzyme was mixed with 0.05 ml of 0.1 M K-Pi buffer pH 6.2 and with 0.2 ml of cofactor stock solns to give the following final concentrations: ATP 3.0 mM; NADPH 0.5 mM; NADH 0.5 mM; FAD 0.5 μM; FMN 0.5 μM; $MnCl_2$ 1.0 mM; $MgSO_4$ 5.0 mM. Prior to adding enzyme, the pH of the mixture of cofactors and buffer was adjusted to 6.2 (with 0.5 M KOH). Then the pH of the reaction mixture was adjusted to 6.9 (with 0.5 M HCL) and the vol. of reaction mixture made up to the designated vol. with H_2O (including vol. of the added mevalonic acid). The incubation mixtures were kept on ice until addition of [^{14}C] MVA to a final concn of 100 μM. [4-^{14}C, 5-^{14}C] MVA lactone (sp. act. 110 mCi/mM) was custom synthesized by Amersham and was converted to free MVA prior to addition by evapg the toluene solvent from the MVA lactone, adding 100 μl (excess) 28% NH_4OH, hydroxide, heating at 50° for 1 hr, and evapg off the NH_4OH *in vacuo*. Reaction vessels (250 ml polypropylene bottles) were shaken at room temp. (*ca* 22°) for 5 hr. At the end of incubation period the reaction mixture was stopped by addition of Me_2CO to give a final concn of 75%, and stored at −80° until used.

To remove the large amount of unconverted MVA and other polar products, the reaction mixture was subjected to charcoal-celite adsorption chromatography. Charcoal:celite (10g:20g) (with a void volume of 50 ml) was used per 100 ml of final dialysed extract, in a 2.5 cm wide glass column. The charcoal-celite was prewashed with Me_2CO and then returned to 0.05 M AcOH. The reaction mixture plus Me_2CO was filtered in a Buchner funnel and the Me_2CO evapd *in vacuo*. The aq. remainder was made 0.1 M in acetic acid and loaded into the column using N_2 pres. at *ca* 70 kPa. The column was washed with 0.1 AcOH (100 ml) 5% Me_2CO (50 ml) and then 100% (3 l). The [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} eluted in the 100% Me_2CO fraction. The 100% Me_2CO fraction was evapd *in vacuo* to *ca* 0.5 ml (aq.–from the 80% Me_2CO remaining in the column). This was transferred to a centrifugal filter tube with a 0.45 μ nylon membrane followed by 2 × 0.5 ml MeCN and 2 × 0.5 ml H_2O rinses of the flask. The sample was filtered and 10 μl injected onto a prep. C_{18} column (1.0 × 15 cm containing Spherisorb 2 ODS) fitted with a 5 ml injection loop, using 40 to 80% 0.1 M aq. MeCN (containing 0.05 AcOH) gradient over 20 min at 4 ml/min. The elution times of the peaks were noted using an on line radioactivity detector (Trace 7140 Packard). The major product was [^{14}C]GA_{12}-aldehyde at R_t 16.0 min with less [^{14}C]GA_{12} at R_t 12.0 min. The entire sample was then run on the HPLC column disconnected from the detector, with the peaks manually collected by retention time directly at the end of the column. The [^{14}C]GA_{12} needed further purification on an analytical C18 column (0.4 × 25 cm), Microsorb, (Rainin) run at 1 ml/min to remove traces of the earlier peak material. The overall efficiency was 6.6% for [^{14}C]GA_{12} aldehyde production and purification, and 2.9% for [^{14}C]GA_{12} (though as only 50% of the MVA would be of the correct isomer, and therefore utilized in the reaction, the conversion from usable MVA would be twice these values).

Extraction and purification of endogenous gibberellins. Pea seed (200 g) was homogenized with an Omni-Mixer in 80% MeOH containing 10 mg/l BHT. [^{14}C]GA_{12} and [^{14}C]GA_{12}-aldehyde (1 μCi each) were added to the homogenate which was stirred overnight at 4°. After filtration the extract was divided into two and evapd to *ca* 50 ml at 33° *in vacuo*. The pH was adjusted to *ca* pH 8 with 0.01 M NH_4OH, and the extract filtered through 2 cm of PVP in a Gooch crucible, followed by a wash with 20 ml H_2O. The filtrate was acidified to pH 3 with HCl, filtered and passed through a charcoal-celite column (15 g charcoal:30 g celite). The column was rinsed with 0.01 N HOAc followed by 1.5 l Me_2CO. The material eluted from the column with 100% Me_2CO was evapd to less than 5 ml *in vacuo* at 33° then centrifuged through a glass fiber prefilter and a nylon 66 membrane filter (pore size 0.45 μm) prior to HPLC.

HPLC. Samples were injected onto an analytical C_{18} HPLC column [5 μm Microsorb 4.6 × 250 mm (Rainin)] using a 5 ml injection loop. The column was eluted with H_2O–MeCN (both containing 0.1 M AcOH) gradient as follows: 0 to 20% over 2 min; 20 to 35% over 15 min; 35 to 75% over 15 min; 75 to 100% over 2 min and holding at 100% MeCN for 5 min before returning, re-equilibration with 0.1 M AcOH and rerunning. The column eluate was passed through the on line radioactivity

added [^{14}C]GA_{12} and [^{14}C]GA_{12}-aldehyde, and the GA_{12} and GA_{12}-[^{14}C]GA internal standards

240(22)	239(4)	225(9)	% ^{14}C = 70.4%
256(24)	255(11)	241*(27)	sp.act. = 351.4 mCi/mMol
240(20)	239(20)	225(17)	% ^{14}C = 51.5%
256(23)	255(11)	241*(37)	sp.act. = 257.1 mCi/mMol
241(46)	239(14)	227(11)	% ^{14}C = 68.5%
257(100)	255(53)	243(17)	sp.act. 342.0 mCi/mMol
241(100)	239(24)	227(17)	% ^{14}C = 24.2%
257(32)	255*(32)	243(5)	sp.act. = 120.8 mCi/mMol

mass/charge ratio. The % ^{14}C and specific activity are calculated from the

monitor with the data recorded as counts per each 12 sec interval at an efficiency of *ca* 10%. Fractions containing radioactive material were collected separately. The radioactive peaks were then rerun on the same column in 55 and 65% aq. MeCN (with 0.1 M AcOH) for GA_{12} and GA_{12}-aldehyde, respectively, and the radio-labelled peaks collected. The labelled peaks were dried down *in vacuo* at 33°, redissolved in 0.3 ml MeOH, methylated with 2–3 vol of ethereal CH_2N_2, dried down under N_2, and subjected to further purification by isocratic HPLC in 70 and 80% aq. MeCN (with 0.1 M AcOH) for GA_{12} and GA_{12}-aldehyde, respectively. The peaks co-eluting with the radio-labelled markers were collected, dried down *in vacuo* at 33°, transferred with MeOH to 100 μl glass tubes held in a larger capped glass vial, and taken to dryness under N_2 and then *in vacuo* over P_2O_5.

GC/MS analysis. GC/MS analyses were done with a Hewlett Packard 5890A gas chromatograph connected to a 5970B mass selective detector and equipped with an HP 9133 computer. Samples were each dissolved in 1.5 μl MeOH and injected (splitless) onto a 12.5 m × 0.2 mm HPI (methyl silicone) bonded phase silica capillary column with a helium flow rate of *ca* 35 cm/sec. After injection, the temp. was maintained at 105° for 1 min, then programmed at 30° min^{-1} to 210°, 4° min^{-1} to 220°, 1.0° min^{-1} to 240° and finally 10° min^{-1} to 275°. The scan mode was used from 100 to 400 mass units at 1.43 scans per sec with an ionizing energy of 70 eV. GA_{12} had an R_t of 10.5 min and GA_{12}-aldehyde had R_t 10.1 min.

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