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Validation of a Radioimmunoassay for Indole-3-Acetic Acid Using Gas Chromatography-Selected Ion Monitoring-Mass Spectrometry[1]

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ABSTRACT

A radioimmunoassay for indole-3-acetic acid (IAA) has been validated by comparison with a physico-chemical assay utilizing gas chromatography-selected ion monitoring-mass spectrometry and 4,5,6,7-tetradeutero-indole-3-acetic acid as an internal standard. The radioimmunoassay provided a reliable estimate of the free IAA content of etiolated corn shoots. However, base hydrolysis of extracts for determination of ester IAA released substances which interfered with the radioimmunoassay. Interference was detected by internal controls and by lack of agreement with the mass spectral method. Interfering compounds could be removed from extracts by chromatography on diethylaminoethyl- and hydroxypropylated (lipophilic) Sephadex G-25. Following such purification the radioimmunoassay estimate of the total (free + ester) IAA content of etiolated corn shoots agreed with the mass spectral method within 2% on the average.

RIA[3] provides a specific method for quantitative determination of compounds of biological interest (4). The sensitivity and simplicity of RIA permit analysis which would be difficult and expensive with other methods. For example, using an RIA for IAA (2), which can detect as little as 1 pmol IAA, it was possible to assay the free IAA content of pea root tips using as little as 0.07 g of plant material (Pengelly and Torrey, unpublished data). However, RIA does not provide unequivocal chemical identification of the compound assayed because the immunologic behavior of interfering compounds may be similar to that of the antigen and, hence, cannot be detected by internal controls (2). Therefore, RIA must be validated by a physico-chemical method which provides proof of structure.

Mass spectrometric analysis of purified samples can provide unequivocal physico-chemical identification. This laboratory has developed GC-SIM-MS as an assay for IAA using d_4-IAA as an internal standard (1). In this paper we report the direct comparison of RIA and GC-SIM-MS methods for quantitating IAA in extracts of *Zea mays* L.

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[3] Abbreviations: RIA, radioimmunoassay; GC-SIM-MS, gas chromatography-selected ion monitoring-mass spectrometry; d_4-IAA, 4,5,6,7-tetradeutero-indole-3-acetic acid; LH-20 Sephadex, hydroxypropylated (lipophilic) Sephadex G-25; PBS: phosphate-buffered saline.

MATERIALS AND METHODS

Plant Material. Seeds of *Zea mays* L. var. Stowell's Evergreen Sweet Corn were soaked for 16 h in cold, running tap water and grown for 4 days in a dark room at 25 C and 90% humidity. Whole shoots or 4-cm shoot tips were harvested under phototropically inactive green light and immediately immersed in acetone chilled with Dry Ice. The amount of acetone was adjusted to give a tissue:acetone ratio of 3:7 (w/v).

Tissue Extraction. The cold tissue-acetone mixture was homogenized in a Sorvall Omni-Mixer, extracted overnight at 22 C in the dark, and filtered. The filtrate was divided into two portions: two-thirds for measurement of free IAA, and one-third for measurement of total (free + ester) IAA. Then 10% of each portion was removed for RIA analysis, and the remaining 90% was used for analysis by GC-SIM-MS. The residual, acetone-insoluble material remaining on the filter paper was dried, weighed, and used as an estimate of the amount of plant material. All data reported here are expressed as $\mu g\ IAA \cdot g^{-1}$ of acetone-insoluble material. For conversion to IAA concentration on a fresh weight basis, dry weight data should be multiplied by 0.085.

GS-SIM-MS. The GC-SIM-MS assay was as described by Magnus *et al.* (1). In brief, a trace amount of IAA-2-^{14}C (51.7 Ci/mol: New England Nuclear) and d_4-IAA at a rate of 50 $ng \cdot g^{-1}$ original fresh weight of tissue for free IAA determination and 300 $ng \cdot g^{-1}$ original fresh weight of tissue for free + ester IAA determination were added to the acetone filtrates, the filtrates concentrated *in vacuo*, and the filtrate for determination of free + ester IAA was hydrolyzed in 1 N NaOH for 1 h at 22 C. The extracts were then acidified, partitioned into diethyl ether, and the ether extracts taken to near dryness *in vacuo* (1). The residue was dissolved in aqueous ethanol, placed on a 2.5 ml bed volume DEAE-Sephadex (Sigma) column, and eluted with a linear gradient of 50% (v/v) aqueous ethanol containing from 0 to 5% glacial acetic acid. Fractions containing IAA (about 30 ml elution volume) were pooled, dried *in vacuo*, and chromatographed on a C_{18}-reverse phase Partisil-10 ODS column (Whatman) eluted with an aqueous solution containing 1% (v/v) acetic acid and 30% (v/v) ethanol. Fractions containing IAA (about 9 ml elution volume) were pooled, dried *in vacuo*, and methylated with diazomethane in ether (3). The methylated product was dried under a stream of N_2 and dissolved in tetrahydrofuran for mass spectral analysis.

Quantitative analysis was with a Hewlett-Packard 5985a quadrapole mass spectrometer/data system coupled to a 3.3 m × 2 mm column of 3% SP-2250, analyzing masses 130 and 134 for base peak of IAA and d_4-IAA, respectively, and masses 189 and 193 for the molecular ion of methyl IAA and d_4-methyl IAA, respectively. The IAA content of the sample was calculated from the dilution of a known amount of d_4-IAA (1). Agreement in calculation of the percent d_4-IAA at 130/134 and 189/193 is within 1

to 2% maximum deviation giving reasonable certainty that no contaminant compounds at these masses emerge at the retention time of IAA. The added IAA-2-^{14}C used to locate IAA during chromatography was carrier-free and did not contribute to the masses examined.

RIA. Approximately 1.5×10^5 dpm of freshly purified IAA-5-^{3}H (27 Ci·mmol^{-1}; Schwarz-Mann) was added to each acetone filtrate for recovery estimates (2), the filtrates were concentrated *in vacuo*, and the filtrate used for assay of free + ester IAA was hydrolyzed in 1 N NaOH for 1 h in the dark. The concentrated extracts were then purified by repeated diethyl ether-buffer partitioning (2) to give a final extract (1–2 ml) in PBS (0.1 M K_2HPO_4, 0.14 M NaCl [pH 8.0]) used directly in the RIA.

In some cases base hydrolyzed extracts were purified further by column chromatography. The ether phase obtained after partitioning was taken to near dryness *in vacuo*, dissolved in aqueous ethanol, placed on a 9 × 0.5 cm DEAE-acetate Sephadex (Sigma) column, and eluted with a 0 to 5% (v/v) linear gradient of acetic acid in 50% (v/v) aqueous ethanol. IAA fractions were pooled, taken to near dryness *in vacuo*, dissolved in 50% aqueous ethanol, placed on a 9 × 0.5 cm LH-20 Sephadex (Sigma) column, and eluted with 50% aqueous ethanol. The IAA fractions were pooled and concentrated *in vacuo*. The aqueous residue was adjusted to pH 8.5 with the addition of 4 ml 50 mM K_2HPO_4 (pH 8.5) and washed by shaking with diethyl ether. The aqueous phase was adjusted to pH 2.5 with 0.28 M H_3PO_4 and extracted 4 times with 2 ml of diethyl ether. The ether fractions were pooled, extracted with 1 to 2 ml PBS, the ether discarded, and dissolved ether removed from the final PBS extract by ebullition with N_2.

Plant extracts in PBS were analyzed for IAA content using a RIA procedure modified from Pengelly and Meins (2). The standard assay mixture consisted of 25 μl IAA-5-^{3}H (30,000 dpm), 150 μl sample in PBS, and 25 μl of anti-IAA antiserum diluted in PBS to give a final antiserum dilution in the assay of 1:200. Tissue extracts were assayed in replicate tubes containing 10, 25, 50, or 100 μl tissue extract and 0, 1, 2, or 4 ng unlabeled IAA as internal standard. Assays of 0, 0.5, 1, 2, 4, 8, and 30 ng IAA in 150 μl PBS were used as external standards. The tubes were mixed and incubated 1 h on ice in the dark. The IAA-antibody complex formed was precipitated with 0.2 ml of saturated $(NH_4)_2SO_4$. The mixtures were incubated 15 min on ice, then centrifuged at 4,000*g* for 15 min at 4 C. Aliquots of the supernatant (0.2 ml) were mixed with 5 ml ACS scintillant (Amersham), salts which are not solubilized allowed to settle, and the samples counted by liquid scintillation. Samples were corrected for quenching, and the amount of IAA in the sample determined by comparing the fraction of ^{3}H bound with the external standard curve.

RESULTS AND DISCUSSION

The quantitative reliability of RIA for estimating the IAA content of plant extracts was determined by comparing RIA with GC-SIM-MS assays. Table I shows such a comparison using acetone extracts of 4 cm shoot tips from 4-day-old, dark-grown corn seedlings. In six experiments the two methods showed good agreement when estimating the free IAA content, although values obtained by RIA were usually higher. The average free IAA content, expressed as μg IAA·g^{-1} dry weight, was 0.40 using GC-SIM-MS and 0.56 using RIA.

In contrast, there was poor agreement between the methods when base hydrolyzed extracts were used to determine total (free + ester) IAA content (Table I). In three experiments, values ranged between 1 and 2 μg IAA·g^{-1} dry weight using GC-SIM-MS; whereas values obtained by RIA were higher and more variable ranging from 4 to 13 μg IAA·g^{-1} dry weight, indicating that substances which interfere with the RIA were released by base hydrolysis. This conclusion was confirmed by assaying increasing volumes of the extract. The same final value will be obtained with different amounts of extract if no interfering substances are present. In the presence of interfering substances, however, an increasing disparity in the final result will be obtained with increasing extract volume due to the increased amount of inhibitor in the RIA. Table II shows results from two experiments using this method. In both experiments the estimate of free IAA content, which agreed well with GC-SIM-MS, did not vary significantly with increased volume assayed, indicating that no interfering substances were present. By contrast, estimates of total (free + ester) IAA content using base hydrolyzed extracts increased with the amount of extract added, and the degree of interference varied between experiments. Therefore, the high and variable estimates of total IAA content obtained by RIA were due to an inhibiting substance(s) released by base hydrolysis.

Table I. *IAA Content in 4 cm Tips of 4-Day-Old, Dark-Grown Zea mays Shoots*

Experiment	IAA Content			
	Free		Free + ester	
	SIM	RIA	SIM	RIA
	μg·g^{-1} dry wt			
1	0.51	0.45	1.85	4.63
2	0.54	0.61		
3	0.40	0.76	1.44	13.30
4	0.41	0.64	1.00	4.90
5	0.28	0.50		
6	0.28	0.41		
Mean[a]	0.40 ± 0.11	0.56 ± 0.13	1.43 ± 0.43	7.60 ± 4.90

[a] Mean values expressed ± SD.

Table II. *Detection of Interference in the RIA Using Different Volumes of Extract*

Extract Assayed	IAA Content			
	Experiment 1		Experiment 2	
	Free	Free + ester	Free	Free + ester
μl	μg·g^{-1} dry wt			
10		3.7	0.49	3.8
25	0.74	5.9	0.41	4.6
50	0.69	7.1	0.46	5.2
100	0.69	8.1		

Such inhibitors were removed with purification of extracts by DEAE- and LH-20-Sephadex chromatography. Table III compares five experiments where the IAA content of whole shoots of etiolated corn seedlings was measured by GC-SIM-MS and RIA. RIA estimates of total (free + ester) IAA content using purified extracts now showed excellent agreement with GC-SIM-MS. Although results were variable from experiment to experiment, reflecting different total IAA content in different lots of plant material, the two methods gave similar values within a single experiment. Estimates of free IAA content determined by the standard RIA method without utilizing chromatographic purification again showed good agreement with GC-SIM-MS results.

The removal of interfering substances from hydrolyzed extracts was confirmed by internal standardization of the RIA (2). With this method a constant volume of extract is assayed with and without known amounts of unlabeled IAA as internal standard. The amount of IAA found by RIA was then plotted as a function of the amount of IAA added as internal standard. With no interfering substances present, this plot yields a straight line with

Table III. *Comparison of RIA and SIM Methods for Determining the IAA Content in Whole Shoots of 4-Day-Old, Dark-Grown Zea mays Seedlings*

Experiment	IAA Content				Slope Internal Standard Curve			
	Free[a]		Free + ester[b]		Free		Free + ester	
	SIM	RIA	SIM	RIA	Slope[c]	% deviation from unity	Slope	% deviation from unity
	$\mu g \cdot g^{-1}$ *dry wt*							
1	0.83	1.00	1.93	1.94	0.92	8	1.00	0
2	0.40	0.57	1.21	1.19	1.03	3	0.98	2
3	0.48	0.69	3.36	4.15	1.05	5	1.07	7
4	0.49	0.44	2.74	2.13	1.06	6	0.90	10
5	0.46	0.45	1.29	1.27	1.09	9	0.91	9
Mean	0.53 ± 0.17[d]	0.63 ± 0.23	2.11 ± 0.93	2.14 ± 1.20		6.2 ± 2.4		5.6 ± 4.4

[a] RIA of free IAA content determined by standard RIA method.

[b] RIA of free + ester IAA content performed on base hydrolyzed extracts purified by DEAE- and LH-20 Sephadex chromatography.

[c] Slope determined by regression analysis of the line, Amount IAA found by RIA *versus* amount of IAA added as Internal Standard, using the least squares method.

[d] Mean values expressed ± SD.

a slope of 1.0 and a *y*-intercept equal to the amount of IAA contributed by the extract. Interference in the RIA will cause a change in the slope of the internal standard curve. Assays showing a slope deviating from unity of more than 15% are rejected. In each experiment we found that slopes of internal standard curves were well within the 15% criterion (Table III) and the average deviation in slope was about 6% for both assays of free IAA (extracts not purified) and free + ester IAA (extracts purified). This verifies the efficacy of chromatography for removing inhibiting substances.

The results reported here demonstrate the importance of validating RIA techniques by independent methods and the importance of using internal controls in the RIA. Although we found that RIA provided a reliable estimate of free IAA, base hydrolysis of extracts to release IAA from its ester forms also released substances which interfered with the RIA. In some cases it was possible to remove inhibition by dilution of the extract (data not shown). Thus, an initial survey using internal RIA controls may give the false assessment that subsequent assays will be free of interference, and internal controls should be routinely employed. In conclusion, we found by direct comparison with a physicochemical method that RIA provides a reliable estimate of both free and ester IAA in extracts of etiolated *Zea mays* shoots.

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LITERATURE CITED

1. MAGNUS V, RS BANDURSKI, A SCHULZE 1980 Synthesis of 4,5,6,7 and 2,4,5,6,7 deuterium-labeled indole-3-acetic acid for use in mass spectrometric assays. Plant Physiol 66: 775–781
2. PENGELLY WL, F MEINS JR 1977 A specific radioimmunoassay for nanogram quantities of the auxin, indole-3-acetic acid. Planta 136: 173–180
3. SCHLENK H, JL GELLERMAN 1960 Esterification of fatty acids with diazomethane on a small scale. Anal Chem 32: 1412–1414
4. YALOW RS 1978 Radioimmunoassay: a probe for the fine structure of biologic systems. Science 200: 1236–1245