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ELISA DETERMINATION OF IAA USING ANTIBODIES AGAINST RING-LINKED IAA

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Key Word Index—Enzyme-linked immunosorbent assay (ELISA); affinity chromatography; auxin; indole-3-acetic acid.

Abstract—An enzyme-linked immunosorbent assay (ELISA) for indole-3-acetic acid (IAA) is described which uses antibodies raised against IAA conjugated to carrier protein on the indolic ring of IAA. As little as 0.5 pmol of IAA is detectable with the ELISA. There is no significant cross-reactivity with amide conjugates of IAA and samples do not need methylation, in contrast to an ELISA using antibodies raised against carboxyl-linked IAA. Affinity chromatography on IAA–agarose was used to purify antibody preparations. Measurements of IAA levels in crown gall tumour tissue lines were made using the assay.

INTRODUCTION

The development of immunoassays has generated considerable interest in their use for measuring plant growth regulators [1]. The specificity and sensitivity of immunoassays, coupled with the relatively low cost of the required equipment, have made these techniques attractive. Although recent reports have demonstrated the need to validate these assays for each tissue type examined and to purify the tissue extracts adequately to remove all interfering materials [2–4], these assays can still compare favourably, in regard to time and cost, with physicochemical measurements of growth regulators.

The quantitation of the auxin indole-3-acetic acid (IAA) has been reported using both radioimmunoassay (RIA) [5, 6] and enzyme-linked immunosorbent assay (ELISA) [7]. The ELISA does not require the use of radioisotopes, but uses an enzyme–antigen conjugate as a tracer, although determination of recoveries using radioisotopes as internal standards is often used. The first RIA for IAA was reported by Pengelly and Meins [5] and used antibodies raised against IAA conjugated to carrier protein on the indolic ring, presumably at the nitrogen (IAA-N). Subsequently, Weiler [6] and Weiler *et al.* [7] described an RIA and an ELISA, respectively, using antibodies raised against carboxyl-linked IAA (IAA-C). Better titres were obtained with the IAA-C antibodies than with the IAA-N antibodies. The IAA-C antibodies, however, showed more cross-reactivity with some conjugates of IAA than did the IAA-N antibodies, although monoclonal IAA-C antibodies have now been reported with reduced cross-reactivity [8]. The use of IAA-C antibodies, however, whether polyclonal or monoclonal, requires the methylation of samples, which adds to sample preparation time and requires the use of hazardous chemicals. Because of the free carboxyl group on the IAA-N antigen, the use of IAA-N antibodies does not require the methylation of samples.

The following results describe the application of the IAA-N antibodies to the technique of ELISA and the use of affinity chromatography to compensate for the low antibody titres obtained with the IAA-N antigen.

RESULTS AND DISCUSSION

Polyclonal antibodies raised against IAA-N–BSA were of primary interest, but similar antibodies against IAA-C–BSA were also raised for comparison. Ammonium sulphate precipitated (crude) preparations of the IAA-N antibody showed some activity in the ELISA (Table 1), but this was low compared to that of the crude IAA-C antibody.

Crude IAA-N antibody was affinity-purified on a column of 10 ml of IAA-N–agarose. Elution with pH 11.6 phosphate buffer gave efficient separation of antibody activity from most of the protein in the crude preparation (Fig. 1). When two halves of a crude antibody sample (250 mg total protein) were chromatographed separately

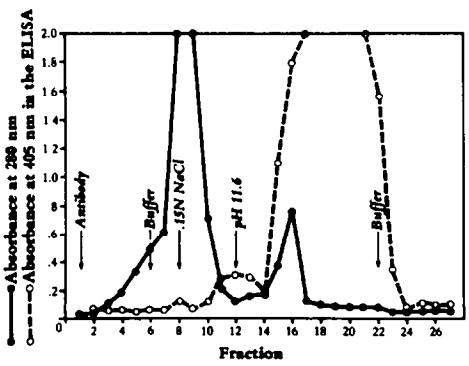

Fig. 1. Elution profile of anti-IAA-N from IAA-N–agarose, starting with approximately 100 mg of antibody. Fraction size = 3–4 ml; flow rate = 0.3–0.5 ml/min. Similar profiles were obtained with anti-IAA-C on IAA-C–agarose, eluted with IAA-Me.

and the most active fractions were combined, 5.8 mg of protein with activity in the ELISA was obtained. This was used at a coating concentration of 0.8 μg/well and provided enough material for coating over 80 microtitre plates. Material which had not bound to the column was rechromatographed on the same column after washing, and active antibody was again eluted with the high pH buffer, indicating that the column had originally been overloaded. The affinity-purified antibody showed greater activity and specificity than the crude preparation (Table 1). Elution with 1 mM IAA was tested with other antibody samples with similar results, but this required extensive dialysis of the eluted antibody before it could be used. Purification of the crude IAA-C antibody on a column of IAA-C–agarose with elution by either the high pH buffer or 1 mM IAA-Me improved the activity and specificity of this antibody in the ELISA as well (Table 1). The use of affinity purification thus provided an alternative to housing and injecting a number of rabbits in an attempt to find an animal producing titres sufficiently high for use in an immunoassay.

Using affinity-purified antibodies, the standard curve for the IAA-N ELISA was linear from 0.5 to 50 pmol. This is comparable to the sensitivities and ranges of the RIAs reported previously for IAA, 1–70 pmol for IAA-N [5] and 1–200 pmol for IAA-C [6]. The IAA-C ELISA, however, has been reported as being more sensitive, with a range of 0.02–50 pmol [7]. Our affinity-purified IAA-C antibodies gave a measuring range of 0.15–50 pmol.

The specificity of the affinity-purified IAA-N antibodies (Table 2) was similar to that reported by Pengelly and Meins [5] for IAA-N antibodies used for RIA. The only significant cross-reactivity in both studies was seen with NAA. There was no significant cross-reactivity with any of the amide conjugates tested, other related indoles or with other auxins which might be used in experimental systems. Affinity-purified IAA-C antibodies were much more cross-reactive, particularly with methylated amide conjugates and indoleacetamide. Polyclonal IAA-C antibodies reported by Weiler *et al.* [7] showed similar cross-reactivities with these compounds, as well as with IBA and NAA. The IAA-C monoclonal antibody reported by Mertens *et al.* [8] showed little cross-reactivity except with IAGly. Thus the specificity of the IAA-N polyclonal antibody compares favourably with that of a monoclonal IAA-C antibody, and may provide greater specificity for free IAA in the presence of auxin conjugates.

The IAA-N ELISA was used to examine the levels of IAA in tomato and tobacco crown gall tumour tissue lines, initiated by *Agrobacterium tumefaciens* strain C-58 on tomato and tobacco. On tomato, this strain produced an unorganized, light-brown, often friable, tumour tissue, whereas on tobacco it produced a shoot-forming, light-green teratoma. Both tissues were maintained in culture without the addition of plant growth regulators.

Acetone–water extracts of the tissues from which the acetone and solids had been removed were designated 'crude' extracts. In spite of the specificity of the IAA-N antibodies as determined from cross-reactivity studies, when crude extracts were assayed, dilution of the extract did not parallel the standard dilution curve, indicating the presence of interfering compounds (Fig. 2). Acidic ether partitioning, however, removed this interference and gave dilution curves which paralleled the standards. Recovery after partitioning, as determined by splitting samples and adding IAA to one half, averaged 66% (Table 3). Measurements of IAA in partitioned samples gave lower values for the unorganized tomato tumours than for the tobacco teratomas (Table 4). The levels of IAA estimated for the tobacco lines were similar to those reported by others using RIA and HPLC fluorescence [5, 9–13].

Although, in this case, partitioning removed some interfering materials from the extracts, parallelism cannot be used as the only criterion for sample purity [5]. Further validation of the IAA-N ELISA readings should incorporate the method of successive approximation as described by Reeve and Crozier [14], to determine the level of sample purification needed. These results should also be compared with an independent method, such as GC/MS [2] or HPLC [4]. Determination of the amount of sample clean-up required should be made for each sample type studied, since tissue differences and different extraction procedures can affect immunoassay results [2, 15].

The IAA-N antibody provides the advantage of specificity which compares favourably with the other antibodies which have been described for IAA-C. The use of the IAA-N antibody, however, also eliminates the need to methylate samples, thus decreasing sample preparation time. Affinity purification of the antibody provides an

Table 1. Comparison of crude and affinity-purified IAA-N and IAA-C antibody preparations in the ELISA

Antibody	Coating concentration (μg/well)	Reaction time (min)	Absorbance in ELISA		% Inhibition by IAA
			Without IAA	With IAA*	
IAA-N					
Crude	10	45	0.551	0.416	25
Affinity purified	0.8	38	1.871	0.249	87
IAA-C					
Crude	10	60	0.710	0.205	71
Affinity purified	1	60	1.917	0.396	80

*IAA was methylated for the IAA-C assay.

Table 2. Comparison of affinity-purified polyclonal antibodies with other antibodies reported for IAA

	Cross-reactivity (%)				
	Polyclonal				Monoclonal
Compound	IAA-N*	IAA-C*	IAA-N†	IAA-C‡	IAA-C§
IAA	100	0.1	100	—	0
IAA-Me	0.3	100	—	100	100
IAAsp	< 0.1	0.4	< 0.1	—	—
IAAsp-Me	—	27	—	6	0.5
IAAla	< 0.1	10	—	—	—
IAAla-Me	—	62	—	—	1.5
IAGly‖	0.2	100	—	—	58
IAPhe	0.1	7	—	—	0.6
IAGlt	0.3	68	—	—	—
IAVal	< 0.1	25	—	—	—
IALeu	< 0.1	7	—	—	—
5-OH-IAA	0.3	< 0.1	0.3	0.3	0.2
IAAmide	< 0.1	17	—	9	1
IAN	< 0.1	0.9	0.04	3	1
I-3-MetOH	< 0.1	3	—	—	—
I-3-EtOH	< 0.1	0.5	—	—	0.04
ILA	< 0.1	< 0.1	—	0.5	0.5
PAA	0.4	< 0.03	0.3	0	0.01
IBA	< 0.1	0.7	0.1	10	1
NAA	22	0.2	25	7	0.1
2,4-D	0.09	0.06	0.4	0	0.01
TIBA	< 0.1	< 0.1	—	—	—
Tryptamine	< 0.1	< 0.1	0.03	0.2	—
Tryptophan	< 0.1	< 0.03	< 0.05	0	0.04

*This paper.
†Ref. [5].
‡Ref. [6].
§Ref. [8].
‖The following compounds were methylated for the IAA-C assays.

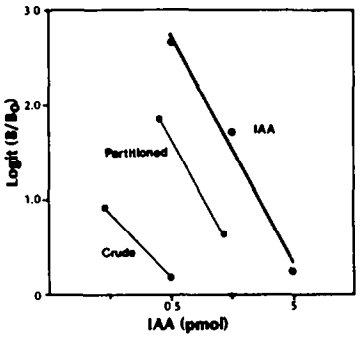

Fig. 2. A comparison of the dilutions of extracts at the crude and partitioned stages (plus added IAA) with dilution of standard IAA. Four extracts were measured at both stages and the results averaged.

efficient means of compensating for the generally low titres produced by the IAA-N antigen. The IAA-N ELISA should thus provide a useful addition to the immunoassays available for the analysis of plant growth regulators.

Table 3. IAA (in ng) in split samples, with added IAA in one half, read at the crude and partitioned stages

Sample	Added IAA	IAA in crude	IAA in partitioned*	Partitioned/crude (%)
1 a		4.0	1.87	47
b	26.3	29.2	18.6	63
2 a		2.8	0.74	26
b	18.9	24.5	15.6	64
3 a		5.6	0.47	8
b	24.7	33.6	17.9	53
4 a		2.0	1.37	69
b	20.8	18.2	15.5	85
5 a		6.8	1.00	15
b	15.9	20.7	9.01	44

*Corrected to 1 ml of crude extract.

EXPERIMENTAL

Chemicals. IAA, bovine serum albumin (BSA), alkaline phosphatase (EC 3.1.3.1) (AP), ω-aminopentylagarose, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased

Table 4. Auxin levels in tomato and tobacco crown gall tumour tissues

Tissue	Growth temperature	No. of samples	IAA (ng/g fr. wt) (± s.e.)
Tomato	27	4	9 ± 4
Tobacco	27	6	21 ± 3
Tobacco	21	4	41 ± 10

from Sigma Chemical Co. Indoleacetylalanine (IAAla), indoleacetylaspartic acid (IAAsp), indoleacetylglutamic acid (IAGlu), indoleacetylglycine (IAGly), indoleacetylleucine (IALeu), indoleacetylphenylalanine (IAPhe) and indoleacetylvaline (IAVal) were gifts from Dr. Roger Hangarter, University of Illinois. Other compounds tested for cross-reactivity, on a molar basis, were 5-hydroxyindoleacetic acid (5-OH-IAA), indoleacetamide (IAAmide), indoleacetylnitrile (IAN), indole-3-methanol (I-3-MetOH), indole-3-ethanol (I-3-EtOH), indolelactic acid (ILA), phenylacetic acid (PAA), indolebutyric acid (IBA), naphthyleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), triiodobenzoic acid (TIBA), tryptamine and tryptophan.

Preparation of antibodies. The indolic nitrogen antigen, IAA-N–BSA, was synthesized using the Mannich reaction [5]. A carboxyl-linked antigen, IAA-C–BSA, was prepared using the mixed anhydride reaction [7]. After dialysis against H_2O, the antigens were lyophilized and stored at −30°. The antigens were mixed with Freund's complete (first injection) and incomplete (subsequent injections) adjuvants and injected into New Zealand White rabbits. Two rabbits were used for IAA-C and gave similar results; one rabbit was used for IAA-N. Bleedings were made from the ear mid-vein, and the serum precipitated with 45% $(NH_4)_2SO_4$. After dialysis against H_2O the protein concentration was estimated by the absorbance at 280 nm. This preparation was designated as the 'crude' antibody preparation, and was stored at 4° or frozen and lyophilized and stored at −30°.

Synthesis of the enzyme tracers. For the synthesis of IAA-N–AP, 1.75 mg IAA was dissolved in 1 ml 0.01 M K-Pi buffer, pH 7.2. To this was added 0.3 ml 3 M NaOAc, and then 0.4 ml 7.5% formaldehyde soln. Buffer (3.2 ml) was added to the soln, followed by 0.5 ml (1000 U) of AP. The soln was then incubated at room temp. with gentle stirring overnight. The procedure used for the synthesis of IAA-C–AP was that of Weiler *et al.* [7]. Enzyme tracer preparations were then dialysed against three 2-l. changes of TBS (50 mM Tris, 1 mM $MgCl_2$ and 10 mM NaCl, adjusted to pH 7.5 with 1 M HCl) at 4°. After testing in the ELISA, they were frozen and stored in aliquots appropriate for one plate each.

Preparation of affinity columns. Crude antibody preparations were further purified by affinity chromatography on columns (1.2 × 8.5 cm) of agarose–IAA. For the synthesis of the IAA-N–agarose, 5 ml ω-aminopentylagarose was washed with H_2O and suspended in a final vol. of *ca* 10 ml. To this was added 3 ml 3 M NaOAc, followed by 4 ml 7.5% formaldehyde, and finally 20 mg IAA dissolved in 3 ml borate buffer, pH 8.5, and neutralized with 0.1 M HCl. The suspension was stirred gently overnight, shaded, at room temp. For preparation of IAA-C–agarose, 50 mg IAA was dissolved in 1.5 ml 95% EtOH and taken up to 25 ml with 0.1 M K-Pi buffer, pH 6.5. Eighty mg of EDC was dissolved in another 25 ml buffer. The two solns were combined and stirred gently for 1 hr. A 55 ml suspension of 20 ml ω-aminopentylagarose in buffer was then added dropwise with stirring to the IAA soln, which was incubated with gentle stirring at room temp. overnight in the dark. In both cases, the substituted agarose was then poured into a column and washed extensively with 0.01 M K-Pi buffer, pH 7.5, after which it was ready for use. When not in use, the columns were stored in buffer containing 0.02% sodium azide.

Affinity chromatography. Crude antibody (100–125 mg) adjusted to pH 7.5–8.0 was applied to the column. This was followed by 0.01 M K-Pi buffer, pH 7.2; 0.15 M NaCl in buffer; and finally buffer without NaCl. Two methods of elution were tested for both the anti-IAA-N and the anti-IAA-C. The first was 1 μM IAA (or IAA-Me in the case of anti-IAA-C), followed by dialysis of the antibodies against H_2O until the bound hapten was released. The second method used 0.1 M phosphate, pH 11.6, to elute the antibodies into tubes containing 1 M Tris, pH 7.0. No dialysis was necessary with antibodies eluted with the second method. The activity of the antibodies was then tested in the ELISA, the active fractions were combined, the preparation was divided into aliquots appropriate for use with one plate each, and the aliquots were stored at −30°.

The ELISA. Antibody was dissolved or diluted in $NaHCO_3$ buffer (50 mM, adjusted to pH 9.6 with 1 M NaOH) and distributed in wells of a microtitre plate (Immulon I, Dynatech), 200 μl/well. The soln was incubated in the wells at 4° overnight and then used for the assay. The immunoassay procedure was based on that described by Weiler *et al.* [7] with four replicates of each sample. The absorbance of the solns at 405 nm was read using a Biotek ELISA Reader. Absorbance readings were converted to the logit $(B/B_0) = \ln (B/B_0)/(1 - B/B_0)$, where B_0 is the maximum binding of the enzyme tracer and B is the binding in the presence of IAA added in the form of sample or standard.

Tissue cultures. Crown gall tumour tissue cultures of tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum* cv Wisconsin 38) were used. Tumours were initiated by *Agrobacterium tumefaciens* strain C-58 and were isolated and grown in culture as described elsewhere [16]. Plant tissues were homogenized with a Tekmar Tissumiser in cold Me_2CO–H_2O (4:1) (5 ml for up to 500 mg tissue, 1 ml/100 mg for more than 500 mg) for 60 sec. The homogenate was then centrifuged for 15 min at 4900 *g*, the supernatant saved and the pellet resuspended in cold 80% Me_2CO and recentrifuged. The supernatants were combined and the Me_2CO removed by evaporation *in vacuo*. The remaining aq. fraction was then frozen at −30°, thawed and centrifuged at 4900 *g* for 30 min. The supernatant was designated the 'crude' extract. For further purification the crude extract was adjusted to pH 3.0 with 0.1 M HCl and was partitioned twice against Et_2O. The Et_2O phases were combined and the Et_2O was evaporated *in vacuo*. The sample was then transferred to a small vial with Me_2CO, which was evaporated under nitrogen. The sample was taken up in H_2O and used for the IAA-N assay. For the IAA-C ELISA, samples were methylated with CH_2N_2 before being taken up in H_2O.

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