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MONOCLONAL ANTIBODIES FOR THE ANALYSIS AND PURIFICATION OF ISOPENTENYLADENINE CYTOKININS

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Key Word Index—*Physcomitrella patens*; Funariaceae; moss; mutants, cytokinins; isopentenyladenine; monoclonal antibodies; immunoaffinity chromatography.

Abstract—A series of monoclonal antibodies (McAb) have been generated which show a high affinity for the cytokinin, isopentenyladenosine (2iPA). The McAb show different specificities to a range of cytokinins in a radioimmunoassay and are suitable for the analysis of isopentenyladenine (2iP) and related compounds in plant extracts. One McAb, MAC 160, has a high cross-reactivity with several cytokinins and is a good candidate for use in group separation or the analysis of a number of cytokinins simultaneously after separation. When coupled to Sepharose 4B affinity support, the McAb can also be used as immunoaffinity matrices to purify and resolve cytokinins from plant extracts. The McAb have been used to demonstrate the presence of concentrations of 2iP in tissues of the moss, *Physcomitrella patens* which exceed those in its culture medium.

INTRODUCTION

The accurate analysis of plant hormones has relied for several years on physicochemical methods such as the relatively sophisticated technique of mass spectrometry. Of necessity, these techniques involve extensive and often lengthy sample purification. In the past few years, there has been an increased interest in the use of antibodies (Ab) and more recently, of monoclonal antibodies (McAb) [1], for the quantification of hormones in plant tissues.

For one of the groups of plant hormones, the cytokinins, interest in the application of immunological techniques originally arose through investigations of their presence in nucleic acids and especially tRNA [2]. Many of the techniques were based on earlier studies concerned with purines and nucleosides [3]. Antisera and McAb have now been raised against representatives of all the major cytokinin species and their use demonstrated and validated for several plant tissues [1, 4]. In addition to their use for hormone quantification, Ab have been employed to produce immunoaffinity matrices for the purification of plant extracts prior to physicochemical analysis [5–7].

The advantages of immunological over physicochemical methods rely mainly on their speed and ease of use [8] which is considerable if large numbers of samples are to be analysed. Furthermore, the use of McAb, as opposed to antisera, has several additional advantages [1, 9], especially in that it provides a potentially unlimited supply of a single defined Ab. Hence, for example, they may be particularly useful in screening for mutants altered in production of cytokinin where a large number of samples needs to be analysed. The only report of McAb against 2iPA has involved their use in the analysis of tRNA in a similar way to the early work with antisera [10], though McAb have recently been used for the analysis of zeatin riboside (ZR) and dihydrozeatin riboside (DHZR) in crown gall tissues [11]. In this paper, the isolation of a number of McAb with different specificities for 2iPA is reported. These McAb have been used in a radioimmunoassay (RIA) for the quantification of 2iP in the culture supernatants and tissue extracts of the wild type (WT) and mutants of the moss, *Physcomitrella patens* and have been coupled to Sepharose 4B to produce suitable matrices for the purification of cytokinins.

RESULTS AND DISCUSSION

Characterization of monoclonal antibodies

Spleen cells, derived from a rat immunized with 2iPA–BSA conjugate, were fused with mouse myeloma cells and hybrid myelomas isolated which secreted antibodies against 2iPA. After cloning and subsequent selection of cells using a radioimmunoassay for 2iPA, five lines were chosen which secreted Ab showing high binding to 2iPA [^{3}H] dialcohol. These McAb, MAC 156–MAC 160, were analysed in more detail. Table 1 presents the characteristics of the anti-2iPA McAb and their binding and Table 2 their cross-reactivities in a RIA using the [^{3}H] dialcohol as tracer.

Variation was observed in antibody class, the affinities of the McAb for iPA, the degree of cross-reactivity with cytokinins other than 2iPA and the detection ranges of the assays. In keeping with several other anti-2iPA Ab [4], several of the McAb showed high cross-reactivity with other non-polar cytokinins such as benzylaminopurine (BAP) and compounds with non-polar side chains e.g. hexylaminopurine. MAC 157, however, showed no cross-reactivity with other non-polar cytokinins and only low cross-reactivity with closely related non-isopentenyladenine cytokinins and, as such, is very similar to some recently isolated mouse anti-2iPA McAb (Eberle, J., personal communication). MAC 159 showed the least

Table 1. Characteristics of isopentenyladenosine McAb

	Antibody name (MAC)				
	156	157	158	159	160
Ig class	IgG2a	IgG2a	IgM	IgG2a	IgG2a
Affinity (10^9 1/mol)	0.86	0.86	0.41	4.4	2.7
RIA—assay range (pmol)	0.3–30	0.2–50	0.5–50	0.1–10	1–50
RIA—50% binding (pmol)	3	3	5	1.5	7
RIA—supernatant dilution	1/30	1/480	1/240	1/300	1/6
RIA—pH	6.5	8	6.5	6.5	8

Table 2. Cross-reactivities* of isopentenyladenosine McAb with cytokinins and related compounds

	MAC number				
	156	157	158	159	160
Competitor					
Isopentenyl adenosine	100	100	100	100	100
Isopentenyl adenine	11	20	23	50	106
Zeatin riboside	1.4	1.6	3.1	0.3	11
Zeatin	<0.1	2.3	2.0	0.3	32
Dihydrozeatin riboside	10	4.1	36	0.8	35
Dihydrozeatin	0.7	3.2	7.1	<0.1	79
Benzyladenine riboside	6.8	1.9	8.4	55	>133
Hexyladenine	>58	<0.1	>733	40	73
Adenosine	<0.1	<0.1	<0.1	<0.1	<0.1
'Anti-cytokinin'†	<0.1	<0.1	<0.1	<0.1	<0.1

*Based on 50% inhibition values in the RIA.

†4-(Cyclo-pentenylamino)-2-methylthio-pyrrolo[2,3-d] pyrimidine.

cross-reactivity with the more polar dihydrozeatin- and zeatin-like cytokinins. Interestingly, MAC 160 showed a high degree of cross-reactivity with all cytokinins and showed high and similar cross-reactivity with cytokinin bases and ribosides, a feature not reported for other McAb. This McAb, therefore, seems highly suitable as a wide range anti-cytokinin Ab for group separation of cytokinins [1, 12]. None of the McAb showed any binding to the non-cytokinin purine, adenosine (Ado), and thus were highly specific for N^6-substituted purines. The affinities were high and similar to those observed for other anti-cytokinin antisera [1] and McAb (Eberle, J., personal communication). In addition, the assay ranges using the dialcohol tracer in a RIA were low, MAC 157 and 159 having minimum detection levels of 0.2 and 0.1 pmol, respectively. In particular, the characteristics of these latter McAb suggested their suitability for the assay of 2iPA or 2iP in plant extracts.

One important feature revealed during the characterisation of the McAb using the RIA was their pH optima. Most assays are carried out at pH's close to neutral [1]. However, one of the Ab described here, MAC 157, was especially sensitive to pH—at pH 8 it bound 15% more radiolabel than at pH 6.5—while, for example, MAC 159 showed maximum binding at pH 6.5. MAC 160 was also best employed in the RIA at pH 8. The other McAb were relatively insensitive to pH over the range pH 5–8. Based on 50% binding values, the sensitivity of the RIA using MAC 157 was increased by a factor of *ca* 4 at the higher pH.

Immunoaffinity chromatography

There are several reports in the literature concerning the use of anti-hormone Ab for immunoaffinity chromatography [5–7, 13, 14]. Antibodies raised against a number of cytokinins have been coupled to a variety of supports including cellulose [5], Sepharose 4B [13] and Glycophase silica [14] to produce immunoaffinity matrices with capacities between 100 ng and 2 μg/ml cytokinin. The bound hormones can be eluted with a variety of disruptive agents though the most convenient for hormone purification appears to be methanol [5, 14]. The bound Ab appear to show unexpected stability in the presence of this solvent [14] though there is a suggestion of some loss in initial activity [5]. Hence, two of the McAb characterised in this study, with differing cross-reactivities in a RIA, were selected and coupled to Sepharose 4B to examine their use as immunoaffinity matrices.

Immunoglobulin from MAC 159 and MAC 160 supernatants was coupled to Sepharose 4B using the CNBr-activated form. The binding of a number of radioactively-labelled cytokinins to those columns was examined. Since there was a suggestion that methanol may harm the

Table 3. Recovery of radiolabelled cytokinins (%) from immunoaffinity matrices

	MAC Number							
	159				160			
	Eluant*				Eluant*			
	0%	10%	30%	50%	0%	10%	30%	50%
2iPA dialcohol	0	0	0	97	0	0	99	0
2iP	0	0	2	94	0	0	0	94
DHZR dialcohol	0	67	33	0	0	50	50	0
DHZ	0	100	0	0	0	2	43	55
Z	0	94	0	0	0	0	91	0
BAP	0	0	0	100	0	0	0	100
Ado	94	0	0	0	100	0	0	0

Abbreviations used have been given in text except: DHZ, dihydrozeatin.
*Percentages indicate either buffer alone (0%) or methanol in water.

matrices [5], the minimum concentration required to remove the bound cytokinin was investigated. For MAC 159 matrix, 50% methanol in water was sufficient to remove 2iPA while MAC160 required only 30%. Fifty percent methanol has also been shown by the authors to be appropriate for eluting the hormone from an anti-abscisic acid Sepharose 4B immunoaffinity matrix [13]. As shown in Table 3, other cytokinins were also found to bind to the matrices. However, the different cytokinins could be removed from the matrices sequentially using increasing concentrations of methanol approximately in the order of their predicted cross-reactivities in the RIA from the data in Table 2. Thus, for MAC 159, 2iPA dialcohol required a higher concentration of methanol for elution than dihydrozeatin riboside (DHZR) dialcohol but the same as BAP and, for MAC 160, cytokinin bases (which cross-react strongly with MAC 160 in the RIA) require the same or a higher concentration of methanol for their elution from the affinity matrix. Cross-reactivities of immunoaffinity matrices with cytokinins other than those against which the Ab was initially raised have not been examined before and the binding and sequential elution of cytokinins described here demonstrates the potential of such columns for both purification and separation of cytokinins.

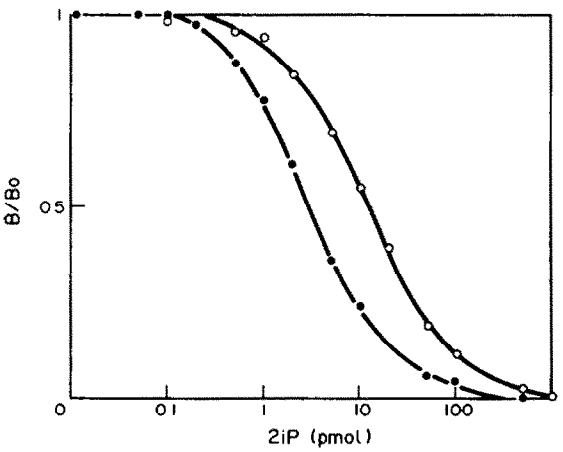

Fig. 1. Standard curves for isopentenyladenine radioimmunoassays. Assays were performed using either MAC159 (closed circles) or MAC157 McAb (open circles), [^{3}H]2iPA dialcohol as tracer and 2iP as the competitor as described in the Experimental. Each point is the mean value from triplicate assays. Bo, amount of radiolabel bound in the absence of unlabelled 2iP; B, amount of label bound in the presence of the different amounts of unlabelled 2iP. Intra-assay variation was 2–5% for the linear measuring range of the assays.

Analysis of moss extracts

The culture medium of the moss, *Physcomitrella patens*, and its mutants which over-produce gametophores (OVEs [15]) contain the cytokinins 2iP and zeatin (Z) [16], the former being by far the major component of the medium. OVE mutants produce at least 100-fold the amount of 2iP found in WT medium [17] though cytokinins have not yet been indentified in the tissues of either moss type. These plants, therefore, are particularly useful for assessing the suitability of anti-2iPA Ab for the analysis of 2iP and have been used previously to compare results obtained with antisera to those obtained by GCMS [18]. Hence mosses were used to test the McAb characterized here and determine whether the criteria that have been outlined by several authors [9, 12, 19] could be met.

RIAs employing both MAC 159 and MAC 157 were used for the analyses. The 2iP standard curves for these RIAs are presented in Fig. 1. The assays had ranges of 0.4–40 and 2–100 pmol respectively. Culture medium from the moss mutant, OVE201, which produces high levels of 2iP [17] was subjected to a series of analyses including sample dilution (Fig. 2a), internal standardization (Fig. 2b) and HPLC (Fig. 2c). Both sample dilution and internal standardization indicated that no interference was present in the medium in that the former showed extract additivity and the latter parallelism to the standard line. Moreover, the HPLC immunohistogram showed a single peak of immunoreactivity co-chromatographing with 2iP. The content of 2iP in OVE201 culture medium was assessed using two batches of medium and a number of different analyses including dilution, internal standardization, immunopurification on the MAC 159 matrix and analysis by the MAC 157 RIA.

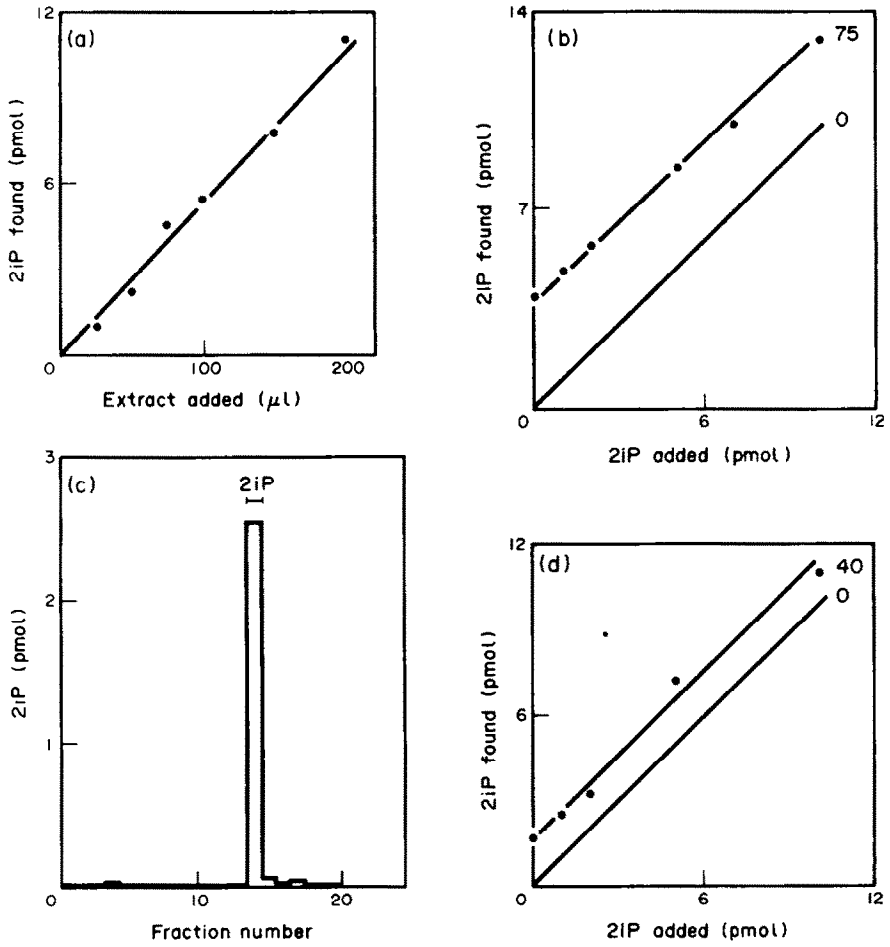

Fig. 2. Analysis of 2iP in moss samples. Analysis of OVE201 culture medium using the MAC159 RIA either by sample dilution (a) where different volumes amounts of extract have been analysed; by internal standardization (b) where different amounts of standard 2iP have been added to a fixed amount of culture medium (75 μl) or by HPLC (c) to resolve immunoreactive material and OVE201 tissue (d) by internal standardization of 40 μl of tissue extract after SepPak purification. Figures (μl) to the right of the lines in (b) and (d) indicate the lines obtained for the sample; zero indicates lines representing the standard curve.

The level of the cytokinin was found to be 55.9 ± 3.6 pmol/ml (total of 9 assays) which was very close to that obtained previously for this mutant—62 nM [17]. The content of 2iP in culture medium of the wild type moss analysed by internal standardization was 0.23 pmol/ml which is also consistent with previous reports [17, 18, 20].

In addition, tissues of both the OVE and WT were analysed using MAC 159. Crude tissue extracts could not be used in the RIA since they showed interference when analysed by internal standardization. The extracts were therefore purified using C_{18} SepPak cartridges which removed the interfering substances (Fig. 2d). The tissue concentration of 2iP obtained from these extracts for OVE201 was 42.5 pmol or 176 nM and for the WT 3.8 pmol or 25 nM. Hence both strains contain 2iP at concentrations greater than those of their respective culture medium. The possible mechanism of 2iP secretion into the culture medium has been discussed previously [18]. The values obtained in this study indicate that passive diffusion as suggested [18] could be operating since the levels in the culture medium detected here have not yet reached equilibrium with the internal tissue concentration. Nevertheless, it is clear that the difference in cytokinin content between OVE and WT cells is not so substantial as between the media—over a 100-fold in the media but only 7-fold between the tissues. This is an interesting observation and raises the possibility that external cytokinin level alone is important for the expression of the OVE phenotype. Analysis, therefore, of the cytokinin content in other mutant tissue is warranted.

EXPERIMENTAL

Chemicals. Radiochemicals were obtained from Amersham International except [^{14}C]Z (11.7 mCi/mmol) and [^{14}C]2iP (11.7 mCi/mmol) which were gifts from Dr R Horgan, UCW, Aberystwyth. DHZR[^{3}H]dialcohol (*ca* 4Ci/mmol) was synthesized as below for 2iPA[^{3}H]dialcohol. Cytokinin standards were obtained from Sigma Chemical Co. Ltd; the 'anti-cytokinin', 4-(cyclopentenylamino)-2-methylthio-pyrrolo[2,3 d] pyrimidine, was a gift from S. Hecht.

Synthesis of isopentenyladenosine–protein conjugates. Essentially the method of ref. [21] was adopted, as modified in refs [22, 23]. Isopentenyladenosine (0.1 mmol) was suspended in 100 μl MeOH and 1 ml of $NaIO_4$ (0.1 mmol) added. The mixture was stirred at room temp. for 30 min and then added dropwise to 2 ml of bovine serum albumin (Sigma, 5 mg/ml, pH 9.5) or keyhole limpet hemocyanin (Calbiochem), maintaining the pH at 9.5 with 5% (w/v) K_2CO_3. After 1 hr, the soln was added to 5 ml $NaBH_4$. The mixture was left at 4° for 1 hr before dialysing extensively against PBS (pH 7.2, 0.15 M). The coupling efficiency was estimated by UV absorption to be 14 mol 2iPA per mol bovine serum albumin and 5000 mol 2iPA per mol keyhole limpet hemocyanin.

Synthesis of isopentenyladenosine [^{3}H]dialcohol. The cytokinin dialcohol was synthesized again essentially according to the method of ref. [22]. 0.01 mmol 2iPA was oxidized as above and the products separated on TLC in $CHCl_3$–MeOH (9:1). 3 μmol of the dialdehyde product (estimated by UV absorption) was added to 50 mCi [^{3}H]$NaBH_4$ in 250 μl EtOH. The mixture was left at room temp. for 30 min and then separated on TLC in $CHCl_3$–MeOH (9:2). The dialcohol, R_f 0.54 (2iPA, R_f 0.73), was eluted from the silica gel with EtOH. The specific activity was calculated to be *ca* 4 Ci/mmol.

Derivation of hybrid myelomas. Hybrid myelomas were derived from a fusion of spleen cells of an immunized female F344 rat with the mouse myeloma NSO [24]. The immunization was initiated by an intramuscular injection of 200 μg of 2iPA-BSA in 200 μl PBS emulsified with an equal vol. of complete Freund's adjuvant. Boosts of a further 200 μg of conjugate were performed subcutaneously on days 21 and 68. Serum was collected 10 days after each boost and monitored for Ab-recognizing cytokinins. The fusion was performed on day 111, 3 days after the final boost of 100 μg of conjugate in 1 ml PBS administered intravenously.

The fusion and subsequent hybridoma selection were performed as described in ref. [24]. After initial screening by a dot immunobinding assay, all positives were screened using a radioimmunoprecipitation assay utilizing 2iPA[^{3}H]dialcohol. During cell expansion and cloning, all assays to detect the presence of Ab recognising 2iPA were performed using the radioimmunoprecipitation assay. Selected lines were cloned on soft agar.

Dot immunobinding assay. The assay was based on the method of ref. [25]. One μl dots of 2iPA-KLH (50 μg/ml in PBS) were applied to nitrocellulose sheets and the remaining free protein sites blocked by incubating with 10% FCS in PBS for 15 min. The sheets were then dried and stored at 4° before use. For the assay, the dots were treated with 5 μl of cell culture supernatant, allowed to dry for 20 min and the sheets washed extensively in PBS. The presence of bound rat Ab was detected by the use of rabbit anti-rat immunoglobulin coupled to horseradish peroxidase [Miles, U.K.].

Radioassays. Cell culture supernatants were screened using a radioimmunoprecipitation assay performed in plastic scintillation vial inserts (Fisons, plc). Each supernatant (100 μl) was added to 400 μl of PBS containing 2000 cpm 2iPA [^{3}H]dialcohol and 5% (v/v) horse serum. The components were mixed and incubated for 90 min at 4°. Immunoglobulins and bound cytokinin were precipitated for 15 min by the addition of 500 μl of satd $(NH_4)_2SO_4$. The mixture was centrifuged at 2500 *g* for 5 min, the supernatant discarded and the precipitate dissolved in 300 μl water prior to the addition of 2 ml scintillation fluid (Packard 299). Vials were counted in a Packard Tricarb liquid scintillation spectrometer.

Cytokinin quantification was performed using a RIA modified from the procedure of ref. [26]. The following were added in sequence to a 1.5 ml Eppendorf centrifuge tube: 200 μl of the appropriate pH buffer (10 mM, containing 0.15 M NaCl), 100 μl 0.5% (w/v)γ-globulins in buffer, 100 μl of standard or sample in buffer (or buffer for total binding), 100 μl 2iPA [^{3}H] dialcohol (*ca* 10000 dpm) in buffer and 100 μl cell culture supernatant suitably diluted in buffer. The components were mixed and equilibrated for 90 min. at 4°. To separate the bound label, 750 μl of satd $(NH_4)_2SO_4$–H_2O (10:1, v/v) were added, the suspension mixed and left at room temp. for 15 min. The mixture was then centrifuged (12000 *g* for 5 min), the supernatant discarded and the pellet washed with 500 μl satd $(NH_4)_2SO_4$–H_2O (1:1, v/v). After recentrifuging the precipitate for 10 min and discarding the supernatant, the pellet was dissolved in 100 μl H_2O and 1 ml of Optiphase RIA (LKB) scintillation fluid added. Samples were counted using the Eppendorf tubes as vial inserts in a Beckman LS7500 liquid scintillation counter.

Characterization of McAb classes. The McAb classes were determined by internal labelling with ^{14}C-lysine and analysis by SDS-PAGE as described elsewhere [24]. IgG subclasses were determined by Ouchterlony immunodiffusion.

Preparation of immunoaffinity matrices. The cytokinin McAb was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia). Briefly, the Ig from the equivalent of 150 ml of cell culture supernatant was pptd twice with $(NH_4)_2SO_4$ and coupled overnight at 4° to acid activated Sepharose (2 g) at pH 8.3. The coupling efficiency was estimated by UV absorption to be 93%. Remaining active sites were blocked with 0.2 M Gly at room temp. and the matrix repeatedly washed at low and high pH and high salt conc. The matrix was stored at 4° in PBS containing 0.02% (w/v) NaN_3.

Sample preparation. Moss tissue was grown and harvested as described previously [16, 27]. Tissue was ground in liquid N_2 in a pestle and mortar and extracted twice in 80% EtOH (5 ml/g fr. wt). The EtOH was removed under a stream of N_2 and the sample dissolved in buffer for direct RIA analysis and affinity chromatography or starting solvent for HPLC. Culture filtrate was adjusted to the correct pH and used directly for the RIA, HPLC or affinity chromatography. Purification of moss tissue, other than by immunoaffinity, was carried out by passing the extract in 70% MeOH through a C_{18} SepPak cartridge (Waters Associates [1]) and collecting the second column vol. (CV) of eluant (recovery *ca* 90%).

Immunoaffinity chromatography. A 2 ml bed vol. column was used for all analyses. The column was equilibrated with buffer at the appropriate pH for the McAb and the samples loaded in the same buffer. The column was then washed successively under gravity with 3 CV buffer, 10%, 30% and 50% MeOH. The bed was recycled by washing again in a minimum of 5 CV starting buffer. To characterize the matrices, *ca* 10000 dpm of radiolabelled cytokinin (^{3}H or ^{14}C) were used as standards.

HPLC. Chromatography was performed using a 10 × 150 mm column of Hypersil ODS (Shandon Southern Ltd). A solvent gradient from 0 to 30% MeCN with an exponent of 0.5 was delivered at a flow rate of 5 ml/min using dual Constametric pumps and a Gradient Master controller (LDC, Stone, Staffs.). The eluant was monitored at 270 nm using a SpectroMonitor III UV Monitor. Samples were applied using a Rheodyne 7125 loop injector. Five ml fractions were collected for further analysis by RIA.

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REFERENCES

1. Weiler, E. W., Eberle, J., Mertens, R., Atzorn, R., Feyerbend, M., Jourdan, P. S., Arnsheidt, A. and Weiczorek, U. (1986) in *Immunology in Plant Science* (Wang, T. L., ed.) p. 27. Cambridge University Press, Cambridge.
2. Hacker, B., Van Vunakis, H. and Levine, L. (1972) *J. Immunology* **108**, 1726.
3. Butler, V. P., Beiser, S. M., Erlanger, B. F., Tanenbaum, W., Cohen, S. and Bendich, A. (1962) *Proc. Soc. Natl Acad. Sci.* **48**, 1597.
4. Wang, T. L. and Horgan, R. (1986) in *Cytokinins—Plant Hormones in Search of a Role* (Horgan, R. and Jeffcoat, B., eds) p. 35. British Plant Growth Regulator Group Monograph 14. BPGRG, Bristol.
5. MacDonald, E. M. S. and Morris, R. O. (1985) *Methods Enzymol.* **110**, 347.
6. Sundberg, B., Sandberg, G. and Crozier, A. (1986) *Phytochemistry* **25**, 295.
7. Mertens, R., Stüning, M. and Weiler, E. W. (1982) *Naturwissenschaften* **695**, 595.
8. Sandberg, G., Ljung, K. and Alm, P. (1985) *Phytochemistry* **24**, 1439.
9. Weiler, E. W. (1986) in *Modern Methods of Plant Analysis* (Linskens, H. F. and Jackson, J. F., eds) Vol. 4, p. 1. Springer, Berlin.
10. Woodsworth, M. L., Latimer, L. J. P., Janzer, J. J., Mc Lennan, B. D. and Lee, J. S. (1983) *Biochem. Biophys. Res. Comm.* **114**, 791.
11. Eberle, J., Arnscheidt, A., Klix, D. and Weiler, E. W. (1986) *Plant Physiol.* **81**, 516.
12. Wang, T. L., Griggs, P. and Cook, S. (1986) in *Plant Growth Substances* 1985 (Bopp, M., ed.) p. 26. Springer, Berlin.
13. Knox, J. P. and Galfrè, G. (1986) *Analyt. Biochem.* **155**, 92.
14. Davis, G. C., Hein, M. B., Chapman, B. C., Neely, B. C., Sharp, C. R., Durley, R. C., Biest, D. K., Heyde, B. R. and Carnes, M. G. (1986) in *Plant Growth Substances* 1985 (Bopp, M., ed.) p. 44. Springer, Heidelberg.
15. Ashton, N. W., Cove, D. J. and Featherstone, D. R. (1979) *Planta* **144**, 437.
16. Wang, T. L., Cove, D. J., Beutelmann, P. and Hartman, E. (1980) *Phytochemistry* **19**, 1103.
17. Wang, T. L., Horgan, R. and Cove, D. J. (1981) *Plant Physiol.* **68**, 735.
18. Wang, T. L., Futers, T. S., McGeary, F. and Cove, D. J. (1984) in *Biosynthesis and Metabolism of Plant Hormones* (Crozier, A. and Hillman, J. R., eds) p. 135. Cambridge University Press, Cambridge.
19. Pengelly, W. L. (1985) in *Plant Growth Substances* (Bopp, M., ed.) p. 35. Springer, Heidelberg.
20. Futers, T. S., Wang, T. L. and Cove, D. J. (1986) *Molec. Gen. Genet.* **203**, 529.
21. Erlanger, B. F. and Beiser, S. M. (1964) *Proc. Soc. Natl Acad. Sci.* **52**, 68.
22. Weiler, E. W. and Spanier, K. (1981) *Planta* **153**, 326.
23. Stollar, B. D. (1980) *Methods in Enzymology* **70**, 70.
24. Galfrè, G. and Milstein, C. (1981) *Methods in Enzymology* **73**, 3.
25. Hawkes, R., Niday, E. and Gordon, J. (1982) *Analyt. Biochem.* **119**, 142.
26. Weiler, E. W. (1980) *Planta* **149**, 155.
27. Ashton, N. W. and Cove, D. J. (1977) *Molec. Gen. Genet.* **154**, 87.