

Rapid Identification of Cytokinins by an Immunological Method¹

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ABSTRACT

A method for rapid identification of bacterial cytokinins has been developed in which cultures are fed [³H]adenine, the cytokinins (including ³H-labeled cytokinins) are isolated by immunoaffinity chromatography, and analyzed by HPLC with on-line scintillation counting. Analysis of *Agrobacterium tumefaciens* strains showed that some produced primarily *trans*-zeatin, whereas others produced primarily *trans*-zeatin riboside. *Pseudomonas syringae* pv *savastanoi* produced mixtures of *trans*-zeatin, dihydrozeatin, 1"-methyl-*trans*-zeatin riboside, and other unknown cytokinin-like substances. *Corynebacterium fascians*, produced *cis*-zeatin, isopentenyladenine and isopentenyladenosine. The technique is designed for qualitative rather than quantitative studies and allows ready identification of bacterial cytokinins. It may also have utility in the study of plant cytokinins if adequate incorporation of label into cytokinin precursor pools can be achieved.

During the past decade, improved methods have been devised for the analysis of phytohormones. Each has specific advantages. GC-MS when used with stable isotopically labeled carriers (23) is specific, precise and sensitive. On the other hand, RIA⁴ or ELISA (26, 27) are fast, very sensitive, and multiple samples can be processed. While each technique offers advantages over the other, the speed, sensitivity, and sample handling capability of the immunological methods are attractive, and they have been widely applied to the analysis of a number of phytohormones with variable degrees of success (15, 21, 28).

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⁴ Abbreviations: RIA, radioimmunoassay; 1"MeZR, 1"-methyl *trans*-zeatin riboside; cZ, *cis*-zeatin; cZR, *cis*-zeatin riboside; DZ, dihydrozeatin; DZR, dihydrozeatin riboside; iP, isopentenyladenine; iPA, isopentenyladenosine; Z, *trans*-zeatin; ZR, *trans*-zeatin riboside.

Unfortunately, direct analysis of phytohormones in unfractionated plant extracts by RIA or ELISA can lead to erroneous results. In some cases, crude extracts may contain compounds which cause nonspecific interference; their presence leads to generation of false positives. For example, Cohen *et al.* (6) showed that ELISA of IAA in even semipurified plant samples led to high estimates of the IAA content. As successively more pure samples were analyzed, the apparent IAA values approached those determined by rigorous mass spectrometric analysis. Nonspecific interference is, however, not the only problem. When several cross-reacting phytohormone species are present, a composite result will be obtained. A case in point is the analysis of bacterially produced cytokinins. The primary cytokinin produced by *Agrobacterium tumefaciens* strain C58 has been shown to be zeatin (9). Small amounts of iP are also present, but not in sufficient quantity to interfere with zeatin-specific RIA of supernatants (20). Zeatin determination by ELISA or RIA is therefore likely to give reasonably accurate results. The same is not true for *Pseudomonas syringae* pv *savastanoi* strains. Some strains secrete Z, ZR, and 1"MeZR and, late in stationary phase, these are present in the medium in approximately equal quantities (14). Since the relative cross reactivities of Z, ZR and 1"MeZR with anti-ZR antibody are approximately 10:50:1 (unpublished data), assays of unfractionated supernatants will not give meaningful data. For most plant samples, where several cytokinins are usually present, the same considerations apply.

The problem can be alleviated by combining immunoaffinity chromatography, to purify the cytokinins from nonspecific interfering substances (13), with HPLC to separate the individual components. RIA or ELISA can then be used to detect each cytokinin in the appropriate HPLC fraction (7, 12). The specificity is improved greatly, individual cytokinins may be detected and their levels measured. Although this approach is suited to quantitation, it does require the assay of numerous fractions derived from complete HPLC gradients and is time-consuming. We now report a method for cytokinin identification which, while it does not permit accurate quantitation, does allow rapid recognition of cytokinins present in a complex mixture. The approach uses [³H]adenine labeling, immunoaffinity chromatography, HPLC, and on-line detection of radioactive cytokinins. The specificity of immunoaffinity selection and HPLC resolution are retained and the method adds information as to biosynthetic origin.

MATERIALS AND METHODS

Strains and Constructs

The strains used in these experiments are listed in Table I. *E. coli* strain HB101(pTZ120) contains the *tzs* gene from *Agrobacterium tumefaciens* strain C58 on the multicopy plasmid pUC18 (3). *A. tumefaciens* strain NT1 is a Ti plasmid-cured derivative of strain C58 lacking the *tzs* gene (25). *A. tumefaciens* strain NT1(pTZ24) contains the *virA*, *virG*, and *tzs* genes from *A. tumefaciens* strain C58 on the plasmid pGA482 (18).

Cell Growth and [³H]Adenine Feeding

All strains were maintained in glycerol at -70°C . Working stocks were grown on solid LB media for *E. coli* strains; 523 medium (8) for *A. tumefaciens*, *Corynebacterium fascians*, and *Pseudomonas savastanoi* strains. Where appropriate, recombinant strains were maintained on media containing either 50 $\mu\text{g}/\text{mL}$ ampicillin for HB101(pTZ120) or 50 $\mu\text{g}/\text{mL}$ kanamycin for NT1(pTZ24). For cytokinin analysis, starter cultures were initiated in the appropriate liquid medium (10 mL) as indicated below and growth was monitored at A_{650} . At a suitable starting density, the cultures were divided into three 2 mL aliquots. To two of these, 10 μCi [³H]adenine was added. Growth of the cultures was monitored on the third aliquot. After 3 to 6.5 h growth (250 rpm) either at 37°C (*E. coli*) or at 27°C (all others), the final A_{650} was measured and the cultures were centrifuged (10,000 rpm, 10 min). Supernatants were transferred to Eppendorf tubes, frozen, and stored at -20°C . The media and times of addition of label for individual organisms were as follows.

1. *E. coli*: M9 medium (pH 7.0). [³H]Adenine (10 μCi) was added at early log phase ($A_{650} = 0.2$), cultures were harvested after 3 h ($A_{650} = 0.8$, mid to late log phase).

2. *A. tumefaciens*: VIMGC medium (containing, per liter, Murashige and Skoog salts (0.5 g), glycerol (10 mL), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (0.3 g), KH_2PO_4 (1.8 g), myoinositol (1.0 g), and casamino acids (Difco, 1 g) (pH 5.7). If acetosyringone was to be added, an aliquot of stock acetosyringone solution (20 mM in dimethylsulfoxide) was dispensed into the cooled medium

to yield a final concentration of 200 μM (18). Starter cultures were grown overnight in the presence or absence of acetosyringone. Inasmuch as it had been determined that cytokinin production by acetosyringone-induced cultures peaked in stationary phase (18), [³H]adenine (10 μCi) was added at late log phase ($A_{650} = 0.3$ – 0.6), and cultures were harvested after 6.5 h.

3. *P. savastanoi*: Cultures were grown on Miller's minimal A medium with 0.2% w/v glucose and 0.05% w/v casamino acids to early log phase ($A_{650} = 0.3$), and then incubated with 10 μCi [³H]adenine for 6.5 h. By this time the cultures were just entering stationary phase ($A_{650} = 0.8$).

4. *C. fascians*: Cultures were grown on Klämbt's medium (10) (pH 5.7) until mid log phase ($A_{650} = 0.5$), [³H]adenine was added and cultures were harvested after 6 h as they entered stationary phase ($A_{650} = 1.4$).

Immunoaffinity Purification of Cytokinins

Monoclonal anti-cytokinin antibodies were linked to cyanogen bromide-activated cellulose (13) using a modification of Kohn and Wilchek's procedure (11) to activate the cellulose. Two antibody clones were employed (24): clone 16 possessed good affinity for hydroxylated cytokinins such as Z and ZR. Clone 12 was capable of binding a wider range of cytokinins including iP, iPA, Z, and ZR. Immunoaffinity columns (in polypropylene syringes) were prepared from 0.5 mL of antibody-cellulose (0.25 mL immobilized clone 16, layered over 0.25 mL immobilized clone 12) and washed with 10 volumes buffer A (40 mM ammonium acetate, pH 6.5) before sample application.

Because different cytokinins have slightly different affinities for the mixed immunoaffinity matrix, it is critical that the matrix be in excess in order to ensure that all cytokinins present in the sample can be bound. The current capacity of these columns is about 500 to 800 ng ZR/mL. All samples were therefore applied at less than these levels to ensure that excess capacity remained.

One milliliter aliquots (or less) of the culture supernatants were centrifuged (14,000 rpm/5 min) to remove residual cells and diluted to 1.3 mL with buffer A. Aliquots (50 μL) of carrier adenine and adenosine (each 10 $\mu\text{g}/\text{mL}$) were added, and the pH adjusted to 6.5 to 7.0 with ammonia. The extracts were applied slowly to the immunoaffinity columns which were washed either under "stringent" or "nonstringent" conditions. The stringent wash comprised (sequentially) 5 volumes buffer A, 3 volumes buffer A + 2% DMSO + 0.5 M NaCl, 10 volumes buffer A + 0.5 M NaCl, and 5 volumes buffer A. The nonstringent wash was simply 10 volumes of buffer A.

After washing, columns were purged with nitrogen and the cytokinins were eluted with 2.5 volumes of methanol. Total radioactivity was measured on aliquots of both wash and methanol prior to the methanol being evaporated *in vacuo* to approximately 25 μL . Because *C. fascians* produces large quantities of orange pigment, the above procedure was modified by the inclusion of a 10 mL column of DEAE cellulose (DE-52, Whatman) ahead of the immunoaffinity column (which was increased in size to 1.0 mL).

Table I. Bacterial Strains

Organism	Genotype	Cytokinin Gene	Reference
<i>E. coli</i>	HB101		4
	HB101(pTZ120)	<i>tzs</i>	3
<i>A. tumefaciens</i>	C58	<i>tzs</i>	3
	B3/73	<i>tzs</i>	1
	M2/73	<i>tzs</i>	1
	NT1		25
	NT1(pTZ24)	<i>tzs</i>	18
<i>P. savastanoi</i>	EW1006	<i>ptz</i>	14
<i>C. fascians</i>	NCPBP 2554 ^a	ND ^b	

^a National Collection of Plant Pathogenic Bacteria, United Kingdom. Accession No. 2554, (Baker LAE 1973). ^b Nature of the gene unknown.

HPLC

Samples were subjected to HPLC on an octadecyl silica column (Altex 5 μm , 250 \times 4.6 mm) using a gradient of acetonitrile (10% to 100% over 40 min) in triethylammonium acetate (40 mM acetic acid adjusted to pH 3.38 with triethylamine) (12). ^3H -labeled compounds were detected by a radioactive flow detector (Radiomatic Instruments, model A200) linked on-line to the HPLC. With these HPLC conditions, the cytokinin standards with the closest retention times (Z and DZ) were separated by 1.2 min with baseline resolution as measured by UV detection at 254 nm. However, the Radiomatic detector integration time was 6 s which causes apparent peak broadening and a decrease in resolution of radioactive species.

RESULTS AND DISCUSSION

The basis for the method is simple. Cells are grown in the presence of [^3H]adenine and the labelled cytokinins are isolated from the culture media and purified by immunoaffinity chromatography on immobilized anti-cytokinin antibody columns. They are then identified by HPLC on octadecylsilica and on-line monitoring of radioactivity in the HPLC effluent. If immunoaffinity prepurification were not used, it was expected that other labeled materials would obscure the radioactive cytokinin peaks. However, because of the specificity of the immunoaffinity procedure, which is capable of purifying cytokinins to analytical homogeneity in a single step (13), it was anticipated that meaningful data could be obtained.

To test the approach, an organism was chosen in which the cytokinin biosynthetic pathway was understood and in which cytokinin production was well characterized. Cytokinin biosynthesis in *A. tumefaciens* strain C58 is catalyzed by the enzyme DMA transferase which condenses dimethylallylpyrophosphate and 5'AMP to give the 5' phosphate of isopentenyladenosine (iPMP) (2). Subsequent hydroxylation of iPMP to ZR 5'-monophosphate, dephosphorylation and deribosylation yield Z, which is excreted into the medium at about 0.05 μM in late log phase cultures (18). Smaller amounts of iP are also produced (20). The DMA transferase enzyme is encoded by a gene, *tzs*, on the *A. tumefaciens* C58 Ti plasmid (3), and the expression of this gene is controlled by phenolic substances such as acetosyringone. When bacteria are grown in the presence of acetosyringone, *tzs* expression, DMA transferase activity and Z synthesis are increased dramatically. The levels of Z in acetosyringone-induced cultures may reach 35 μM (18).

A. tumefaciens strain C58 was therefore grown either in the presence or absence of acetosyringone and incorporation of [^3H]adenine into cytokinin was determined. The results are illustrated in Figure 1, A and B. In the absence of acetosyringone, the immunoaffinity-purified culture supernatants were found to contain two ^3H -labeled compounds which co-chromatographed with Z (14.4 min) and iP (35.4 min). That these compounds are, in fact Z and iP, is based on their coelution with Z and iP, respectively, their retention by anti-cytokinin antibody columns, and by the incorporation of adenine. A control experiment in which the plasmidless strain NT1 was grown in the presence of [^3H]adenine is illustrated in Figure

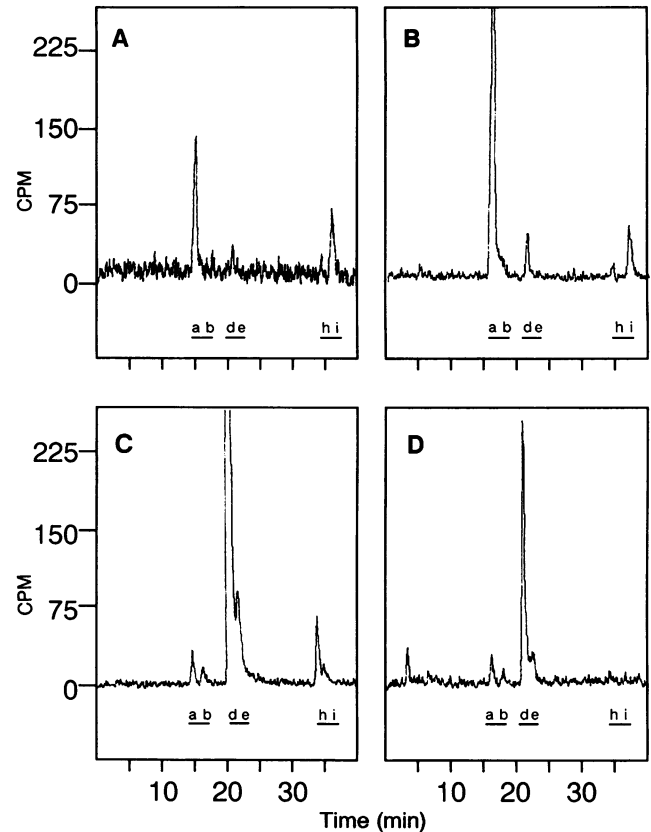


Figure 1. Cytokinin production by *A. tumefaciens* strains. Cultures (2 mL) were labeled with 10 μCi [^3H]adenine and the supernatants were purified by immunoaffinity chromatography on anti-cytokinin antibody columns. Eluted radioactive cytokinins were separated by HPLC and detected by on-line liquid scintillation counting. Retention times of cytokinin standards are indicated by horizontal bars: a, Z; b, DZ; c, cZ; d, ZR; e, DZR; f, cZR; g, 1''MeZR; h, iPA; i, iP. A, *A. tumefaciens* strain C58, 1000 μL , *tzs* gene expression not induced. B, *A. tumefaciens* strain C58, 100 μL , *tzs* gene expression induced by 200 μM acetosyringone. C, *A. tumefaciens* strain B3/73, 1000 μL , *tzs* gene expression induced by 200 μM acetosyringone. D, *A. tumefaciens* strain M2/73, 1000 μL , *tzs* gene expression induced by 200 μM acetosyringone.

2D. Other than a very small peak of iP (possibly originating from tRNA turnover), no cytokinins were detected.

It is important to note that the technique is not designed to be quantitative; it is primarily for identification. However, it did allow measurement of induction of *tzs* expression by acetosyringone. Not only was the expected increase in radioactivity in Z (15.1 min) observed (Fig. 1B), but radioactivity increased also in iP (36.2 min), in ZR (20.5 min), and in iPA (33.8 min). There was a 50-fold increase in incorporation of label into Z and a 10-fold increase into iP. No significant amounts of other radioactive species were present. Clearly the method not only allows detection of known cytokinins quickly and easily but it also allows major changes in their amounts to be observed.

Application of the procedure to other *A. tumefaciens* strains revealed that their pattern of cytokinin secretion differed from

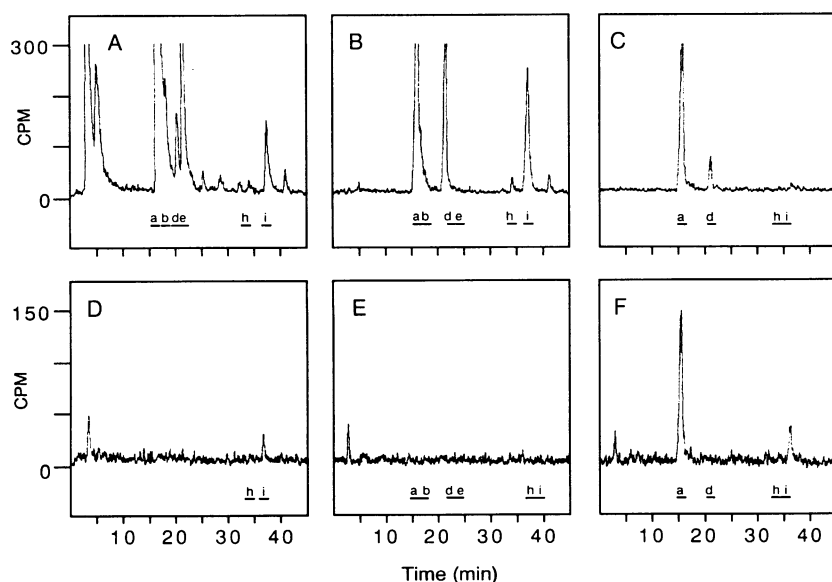


Figure 2. Cytokinin production by native and recombinant *A. tumefaciens* and *E. coli* strains. Cultures were labeled as described in Figure 1. Aliquots of the cell-free supernatants were analyzed either without purification (A) or directly after immunoaffinity chromatography (B–F). Eluted radioactive cytokinins were fractionated and analyzed by HPLC and on-line liquid scintillation counting. Retention times of cytokinin standards are as indicated in Figure 1. A, *A. tumefaciens* NT1(pTZ24): 100 μ L unpurified culture supernatant, *tzs* gene expression induced by 200 μ M acetosyringone. B, *A. tumefaciens* NT1(pTZ24): 1000 μ L immunoaffinity-purified culture supernatant, *tzs* gene expression induced by 200 μ M acetosyringone. C, *A. tumefaciens* NT1(pTZ24): 10 μ L immunoaffinity-purified culture supernatant, *tzs* gene expression induced by 200 μ M acetosyringone. D, *A. tumefaciens* NT1: 1000 μ L immunoaffinity-purified culture supernatant. Culture was grown in the presence of 200 μ M acetosyringone. E, *E. coli* HB101: 1000 μ L immunoaffinity-purified culture supernatant. F, *E. coli* HB101(pTZ120): 1000 μ L immunoaffinity-purified culture supernatant.

that of C58. Two wild-type *A. tumefaciens* nopaline strains B3/73 and M3/73 were examined. Both were known by Southern hybridization to contain homologs of the C58 *tzs* gene (data not shown) and both were shown by ELISA to produce significant levels of zeatin-like cytokinins. The results of [3 H]adenine feeding indicated that strain B3/73 produced labeled compounds which co-chromatographed with Z (14.2 min), DZ (16.0 min), ZR (20.0 min), DZR (21.3 min), and iPA (33.2 min) (Figure 1C). Significantly, most incorporation of label was into cytokinin ribosides rather than into free bases. Strain M2/73 displayed a similar profile. Most radioactivity was present in zeatin riboside (20.5 min), with lesser amounts in Z (15.6 min), DZ (17.4 min), and DZR (21.9 min). In this organism, no iPA or iP were detected. Again, even though the technique is not designed to produce accurate quantitative comparisons, it can be used to make rough estimates of production. Evidently also these *Agrobacterium* strains differ from C58 in that they are deficient in the ribosidase which allows conversion of ZR to Z. The distribution of ability to secrete ZR versus Z in wild-type *A. tumefaciens* strains is of interest from the perspective of early tumorigenic events incited by these bacteria. The technique provides a rapid means of screening the organisms for such information.

To determine the necessity for an immunoaffinity step, the immunoaffinity column was eliminated and a comparison was drawn between the results obtained in its presence and absence. A recombinant *A. tumefaciens* strain, NT1(pTZ24) was used for these experiments. It contains the *virA*, *virG*, and *tzs* genes from *A. tumefaciens* strain C58 in the NT1 chromosomal background and can be induced with acetosyringone. When induced, it excretes high levels of zeatin-like cytokinins into the culture medium (18). A comparison of the HPLC traces of culture supernatants before and after immunoaffinity purification is illustrated in Figure 2, A to C.

HPLC of crude culture supernatant without benefit of immunoaffinity purification, led to detection of numbers of radioactive compounds. Some were very polar, others had retention times close to those of authentic cytokinins. As expected, [3 H]adenine was incorporated not only into cytokinins but into other materials as well. Immunoaffinity purification eliminated both polar and less polar contaminants leaving the cytokinin bases and nucleosides Z, ZR, iP, and iPA as the only radioactive species present (an additional small peak of activity was present at 42 min, its identity is not known).

The technique worked well for other bacteria capable of cytokinin biosynthesis. *E. coli* HB101(pTZ120) which contains the *tzs* gene and is known to secrete mainly Z (3), gave good incorporation of [3 H]adenine into Z (15.2 min) and iP (36.2 min) (Fig. 2F). *E. coli* HB101 cells lacking the *tzs* gene showed no incorporation (Fig. 2E). *Pseudomonas syringae* pv *savastanoi* strain EW1006, which secretes Z, ZR, and 1''MeZR (14), exhibited a multiplicity of radioactive species in the crude culture supernatant (Fig. 3A). Application of immunoaffinity chromatography with the usual "stringent" wash of the matrix, gave only radioactive Z (Fig. 3B, 15.8 min). However if the immunoaffinity matrix was washed under less stringent conditions (Fig. 3D), radioactivity was observed also in Z, DZ, 1''MeZR (26.9 min), and at 24.3 min (possibly 1''MeZ).

Finally, several strains of *C. fascians* were examined. Figure 3D shows the data obtained for strain NCPPB 2554. By utilizing a large quantity of the broad range clone 12 antibody in the immunoaffinity column, it was possible to detect label primarily in iP but also in iPA and cZ. *C. fascians* has been variously reported (Murai *et al.* [16] and references cited therein) to secrete cZ, cZR, iP, iPA, and a range of 2-methylthio cytokinins. Rathbone and Hall (19) reported that only [3 H]iP was formed when cultures were supplied with

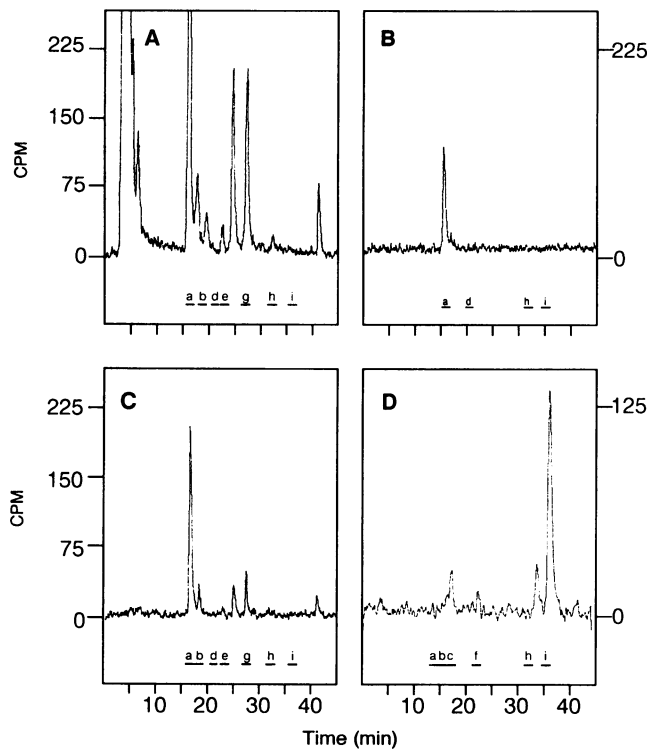


Figure 3. Incorporation of [^3H]adenine into cytokinins produced by *P. savastanoi* and *C. fascians*. Cultures were labeled as described in Figure 1. A, *P. savastanoi* strain 1006: 100 μL unpurified culture supernatant. B, *P. savastanoi* strain 1006: 100 μL immunoaffinity-purified culture supernatant, stringent wash. C, *P. savastanoi* strain 1006: 100 μL immunoaffinity-purified culture supernatant, nonstringent wash. D, *C. fascians* strain NCPPB 2554: 10 mL immunoaffinity-purified culture supernatant, nonstringent wash.

[^3H]adenine. The data reported here favor the conclusion that iPA and cZ can also be labeled. It would be interesting to repeat the same experiments with cZ-specific antibodies.

In summary, the method provides a rapid and efficient way to determine which cytokinins are synthesized by an organism. The degree to which it can be applied to plant cytokinin biosynthesis remains to be determined. In principle there should be little difficulty. Free cytokinin bases and ribosides should be labeled and detected as in bacteria. Cytokinin side chain glucosides do not react with available antibodies but can be detected after glucosidase treatment. The crucial limitation is likely to be the extent to which adenine can be incorporated into the cytokinin precursor pool. In studies of crown gall lines (which overexpress the *A. tumefaciens ipt* gene and produce elevated levels of ZR) we have found good incorporation of [^3H]adenine into cytokinins (unpublished data). Thus, adenine can be incorporated into the AMP pool and thence into cytokinins. Reports in the literature (17, 22) are consistent with these data. The extent to which cytokinin biosynthesis can also be detected in untransformed plants is uncertain. There is one report of incorporation of label into cytokinins (5) but the incorporation was low. Experiments designed to answer this last question are currently in progress.

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