Phytochemistry, Vol. 27, No. 6, pp. 1583–1587, 1988.
Printed in Great Britain.

0031-9422/88 $3.00+0.00

DIHYDROZEATIN RIBOSIDE FROM *PHASEOLUS* SAP: QUANTIFICATION BY RADIOIMMUNOASSAY AND GC-MS

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(*Received* 1 *October* 1987)

Key Word Index–*Phaseolus vulgaris*; Leguminosae; bean; radioimmunoassay; thermospray liquid chromatography-mass spectrometry; cytokinin; dihydrozeatin riboside.

Abstract—Dihydrozeatin riboside, has been identified as the major cytokinin in the sap of decapitated bean (*Phaseolus vulgaris*) plants by combined thermospray liquid chromatography-mass spectrometry. A radioimmunoassay has been developed for the quantification of the cytokinin based on an antiserum raised against a dihydrozeatin riboside-bovine serum albumin conjugate. This antiserum had a high titre, specificity and affinity for dihydrozeatin cytokinins. By the use of internal standardisation, dilution assays, immunohistograms and comparison with GC-CIMS, the radioimmunoassay has been shown to be applicable to the quantification of dihydrozeatin riboside in bean sap.

INTRODUCTION

The bean plant, *Phaseolus vulgaris L.*, has been used extensively for studies on endogenous cytokinins and cytokinin catabolism and conjugation [1–7]. It is perhaps the best characterized higher plant with respect to cytokinin content; a total of seven cytokinins in roots, stems and leaves have previously been identified by MS. Although the normal tissues of the plant contain very little cytokinin, modification of the plant by the production of leaf cuttings or by decapitation above the primary leaves, leads to the accumulation of relatively high levels of cytokinins in the leaves [1,2,6]. This has aided the identification of many of the cytokinins. In decapitated plants, the main cytokinin accumulated is dihydrozeatin-*O*-glucoside (DHZOG) [6] which is considered to be a storage form of cytokinin; outgrowth of the axillary buds leads to a loss of the cytokinin in the lamina [2,6]. The cytokinins in the sap of this system, which are the probable precursors for the lamina cytokinins, have not been unambiguously identified though Palmer and Wong [4] have presented evidence for the presence of at least eight cytokinins in the sap of whole plants.

To date, analysis of cytokinins in beans has been carried out mainly using physicochemical methods and has required extensive purification of large quantities of plant material. The low levels of these substances in many of the tissues, however, indicate that immunological techniques may be useful. Use of radioimmunoassays (RIAs) and enzyme immunoassays (ELISAs) for cytokinin quantification is becoming widespread [see 8 for a review] and, with the correct validation, has produced useful data. Hence, the cytokinins in the sap of decapitated bean cuttings have been examined using both MS and a RIA based on an antiserum against dihydrozeatin riboside (DHZR). A relatively new MS technique—that of thermospray (TSP) LC/MS [9–11]—has also been investigated for the analysis of cytokinins. This technique offers the possibility of identification and quantification of cytokinins without entailing the production of volatile derivatives and the concomitant losses. Furthermore, it avoids the problems of instability to GC of some derivatives [12].

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RESULTS AND DISCUSSION

Cytokinins from sap

Sap (40 ml), harvested from cuttings of *P. vulgaris*, was purified by trace enrichment and a small aliquot examined for immunoreactivity in a RIA for DHZR (see below). Fractions from the regions at which dihydrozeatin (DHZ) and DHZR eluted were pooled (pooled samples S1 and S2 respectively) and then analysed directly by TSP LC/MS under CI conditions as outlined in the Experimental. Quasi-molecular ions $[MH]^+$ for DHZ (*m/z* 222), DHZR (*m/z* 354), zeatin (Z) (*m/z* 220) and zeatin riboside (ZR) (*m/z* 352) were monitored initially, together with the total ion current, since these ions are normally the most abundant in such spectra [13]. A cross-scan report indicated the presence of ions *m/z* 352 and 222 in S1 and *m/z* 354 and 352 in S2 indicating the possible presence of DHZ, ZR and DHZR in sap; ZR having been split between the two pooled samples as would be expected from its elution position on HPLC.

Full spectra were obtained from scans which contained ions indicating the presence of cytokinins. Sample S2 contained a compound with the same R_t as authentic DHZR (5.7 min) whose major ions were at *m/z* (rel. int.) 354 $[MH]^+$ (6), 222 $[BH]^+$ (100) and 202 and a compound with the same R_t as ZR (5.0 min) whose major ions were at *m/z* (rel. int.) 352 $[MH]^+$ (6), $^+$ 220 $[BH]^+$ (100) and 200 (63). In both instances, the ions were identical to the major ions in the spectra of the authentic compounds but the quasi-molecular ion was of lower relative intensity. (Full spectra have been published elsewhere [14].)

The small amounts of the endogenous cytokinins available relative to the standards may explain the lower intensities of these ions. Similarly, the intensity of the quasi-molecular ion of the ribosides was of lower intensity than the spectrum of the respective base. A loss in TSP sensitivity toward the parent ions with respect to the base has frequently been observed for compounds with thermolabile groups, such as the ribosides, and may account for the variability that was obtained in this instance and in subsequent runs [14]. Impurities in the sample affect the effectiveness of the TSP interface and decrease the ion intensities [14] which may also explain the lower intensities in the spectra of the isolated compounds. Hence, under the conditions employed here, which were optimised for the MS and not for the cytokinins, the ribosides were always less stable than the bases. However, further refinement of the technique may improve their stability. Nevertheless, on the basis of their R_ts on HPLC and the major ions in their spectra, sample S2 from *P. vulgaris* sap contained both DHZR and ZR. Based on the intensities of the $[BH]^+$ ions, DHZR was the major cytokinin in the sap of *P. vulgaris* cuttings.

Spectra obtained from an analysis of sample S1, although containing the major ions for Z, ZR and DHZ at the correct R_ts for these compounds, were poor and contained many ions some at a higher intensity and m/z value than the relevant MH^+ ions. Thus, while the presence of Z and DHZ was indicated, they could not be identified unambiguously in bean sap.

This, the first use of TSP LC/MS to identify cytokinins in plant extracts, indicates that the technique may be extremely useful for compounds whose analysis has hitherto relied on the production of volatile derivatives for GC. This may be especially so for some cytokinins which are not very amenable to this latter approach *viz* the cytokinin glucosides [12]. Relatively little purification was required in the current investigations and refinement of the technique, with regard to the TSP interface and LC conditions, allowing a wider range of solvents and chromatographic conditions to be used, should mean that the technique could be applied to a wide variety of plant extracts.

Radioimmunoassay for dihydrozeatin riboside

Dilution of antiserum obtained from rabbits immunized with a DHZR-bovine serum albumin conjugate as described in the Experimental indicated a high titre of antibodies against DHZ cytokinins. Figure 1 shows the dilution curves for this antiserum when assayed in a RIA using either [^{3}H] DHZ or [^{3}H] DHZR as the assay radiotracer. The antiserum bound 50% of the tracer riboside at a dilution of 1/4000 giving a final assay dilution in 600 μl of 1/24 000. The antiserum was stable over a wide pH range showing no decrease in binding from pH 4.5 to 8. It was also stable at either room temperature or 4° for up to 2.5 hr. Quantification of DHZR in an optimized assay at pH 6 using [^{3}H] DHZR as a tracer, gave a standard curve (Fig. 2) with only a small inter- and intra- assay variation. Table 1 summarizes the characteristics of the assay.

When a series of cytokinins and related purines were substituted for DHZR in the assay, the antiserum showed a strong specificity for DHZ cytokinins (Table 2) which compared favourably with a recently reported ELISA, based on a McAb [15], and was superior to other anti-

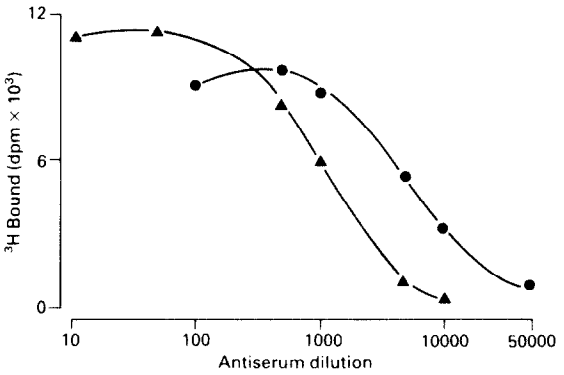

Fig. 1. Binding curves for anti-DHZR antiserum with [^{3}H] DHZ and [^{3}H] DHZR. Dilutions of antiserum were incubated with *ca* 10 000 dpm of either [^{3}H] DHZ (▲) or [^{3}H] DHZR (●) as described in the text and the bound counts determined after precipitation.

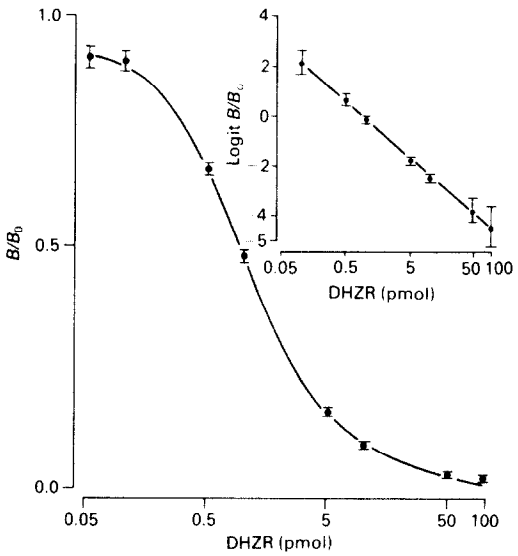

Fig. 2. Standard curves for the DHZR radioimmunoassay. The assay was performed using a 1/24 000 dilution of antiserum, DHZR as competitor and enzymically prepared [^{3}H] DHZR as tracer as described in the Experimental. Inset shows a logit plot of the standard curve. B, amount of radiolabel bound in the presence of different amounts of unlabelled DHZR; B_0, amount of radiolabel bound in the absence of unlabelled DHZR. Vertical bars equal twice the standard error of the mean.

Table 1. Characteristics of anti-dihydrozeatin riboside assay

Final antiserum dilution	1/24 000
Antiserum affinity (1/mol)	3.1×10^9
Measuring range (pmol)	0.3–10.0
Sensitivity (pmol)	0.2
Interassay variance (%)*	9.0
Intraassay variance (%)†	5.6
50% binding pmol	1.0
pH	6

* Five assays.
† Triplicates.

Table 2. Cross-reactivities* (%) of anti-DHZR antiserum with cytokinins and related purines compared to published antibodies

	Antibody		
	This study	[15]	[16]†
Competitor			
Dihydrozeatin riboside	100.0	100.0	100.0
Dihydrozeatin	23.2	67.4	59.7
Zeatin riboside	7.8	1.7	16.1
Zeatin	0.4	1.7	1.3
Isopentenyladenosine	1.8	1.0	3.8
Isopentenyladenine	0.5	1.0	2.0
Dihydrozeatin-*O*-glucoside	0.5	9.7	—
Dihydrozeatin-*O*-glucoside riboside	0.1	0.7	—
Benzyladenine	0.9	—	63.9
Adenosine	0.0	0.0	0.0
Adenine	0.0	0.0	0.0

*Based on 50% inhibition values in the RIA.
†Recalculated to give dihydrozeatin riboside a value of 100%.
—, Not determined.

sera which cross-reacted strongly with BAP [16]. The antiserum had no cross-reaction with adenine or adenosine and only low cross-reactivity with other cytokinins lacking the saturated N^6-side chain; ZR at 7.8% being the most significant. This assay, therefore, appeared suitable for the measurement of small amounts of DHZR in plant extracts. The cross-reactivity has been found to be altered by the use of different tracers [17] which means that, by replacing DHZR with other tracers, the assay may also be suitable for use with other closely related cytokinins.

Quantification of dihydrozeatin riboside in sap

To examine whether the RIA was suitable for the analysis of cytokinins in bean sap, it was necessary to carry out several checks [17, 18]. Samples of sap were serially diluted, or added to known amounts of standard DHZR, or separated on HPLC, or chromatographed on HPLC and added to known amounts of standard and then analysed in the DHZR-RIA. The diluted sap produced a line parallel to the standard curve (Fig. 3a). Similarly, internal standardization of either crude sap or HPLC purified sap produced lines parallel to the standard line (Fig. 3b) as indicated by a *t*-test on the regression coefficients of the lines. These analyses indicated that the sap did not contain substances interfering with the assay. The immunohistogram obtained from sap separated by HPLC did show, however, the presence of small amounts of cross-reacting material in the region of DHZ (Fig. 3c).

Final validation of the assay was performed by quantifying DHZR in portions of the same sample of sap (from 8 plants) by both a physico-chemical method (GC-CIMS) and the DHZR-RIA. One portion of sap was serially diluted and its cytokinin content estimated using the RIA. The value obtained from four triplicate estimates was 268.5 ± 9.2 pmol/ml. Another portion (2.4 ml) of sap was then used to estimate the DHZR content using [U-^{15}N] DHZR as an internal standard for recovery. This standard was synthesized enzymatically from [U-^{15}N] DHZ [19]. One hundred ng of the internal standard was added to the sample which was then purified by HPLC. Following permethylation, the sample was analysed by MIM GC-CIMS on a capillary column. Ions were monitored at *m/z* 424 and 428 which were the characteristic $[MH]^+$ for DHZR-Me and its isotope respectively. A series of standards each containing 100 ng of the synthesised [U-^{15}N] DHZR were also monitored. The calibration line obtained by calculating the ^{14}N:^{15}N ratio for this series is shown in Fig. 4 and was very similar to those obtained for other ^{15}N-labelled internal cytokinin standards [19]. By comparison of the same ratio for the sap sample with the standard line, a value of 258.7 pmol/ml was obtained which is in close agreement with that obtained from the DHZR-RIA demonstrating the suitability of the RIA for the analysis of DHZR in *P.vulgaris* sap. The difference between the two techniques can be accounted for by the cross-reactivity in the region at which DHZ and ZR elute on HPLC (Fig. 3c).

Studies on the sap from intact plants by Palmer and Wong [4] identified several cytokinins on the basis of chromatographic evidence and the presence of molecular ions. The data obtained here, however, indicate that the decapitated bean cutting system differs from the intact plant in that DHZR is the main cytokinin while ZR and, to a much smaller extent, probably DHZ and Z, are also present. Although, the presence of ribotides in the sap would not have been detected in the present study, no evidence was found for glucosides. The differences in the two systems may be the reason for the differences in the cytokinins found in that whole plants were used by Palmer and Wong and these were decapitated above the cotyledonary node thus leaving a significant amount of stem tissue. The stem is known to contain substantial quantities of cytokinin and to be capable of metabolizing

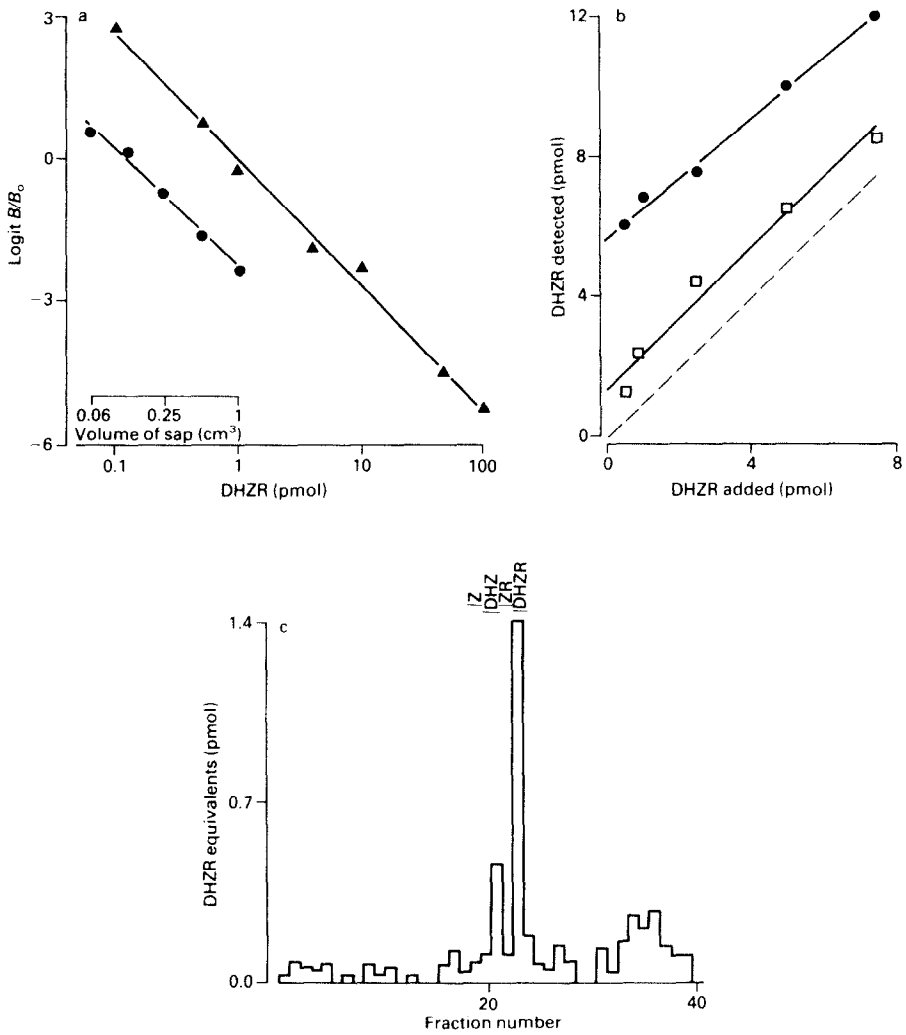

Fig. 3. RIA analysis of *Phaseolus* sap. *Phaseolus* sap was analysed using the DHZR RIA (a) after dilution (●) and compared to the standard line (▲), (b) after the addition of different amounts of unlabelled DHZR to a fixed amount (20 μl) of crude (●) or HPLC (■) purified (equivalent to 4.3 μl crude sap) sap and compared to the standard line (--) or (c) after analysis by HPLC to resolve immunoreactive material (horizontal bars indicate the elution position of the named standard compounds).

applied cytokinins [3]. Hence, cytokinins detected earlier but not detected here may be due to the presence of the stem tissue. The decapitated bean cutting system accumulates relatively large quantities of DHZOG in the leaf blades [2, 6]. Thus, it seems likely that, in this system, DHZR is transported to the leaves where it is deribosylated and glucosylated for storage [2, 3, 6]. The small amounts of DHZR (rather than ZR) which have been identified in the leaves [7] support this hypothesis.

EXPERIMENTAL

Plant material. Seeds of *Phaseolus vulgaris* L. cv. Canadian Wonder were germinated in trays of compost and grown in a glasshouse until the primary leaves were fully expanded. After 3 weeks cuttings were taken which consisted of stem and primary leaves only; all apical and axillary bud tissue was removed. These cuttings were placed in IBA (10 mg/l) for 24 hr in a mist propagator. The cuttings were then rinsed and placed in nutrient soln to develop adventitious roots. Once roots appeared the plants were transferred to a 2 l plastic container (8 plants per container) containing aerated Long Ashton nutrient soln [20] and grown in a greenhouse at 20° under long days. All axillary bud tissue was removed as necessary. Sap was collected under toluene using sterilized silicone rubber tubing attached to the stump of the plants cut just above the container lid.

Preparation of antiserum. New Zealand White rabbits were injected subcutaneously with 1 mg of the cytokinin-bovine serum albumin conjugate (prepared as described previously for isopentenyladenosine [21]), emulsified with complete Freund's adjuvant, on days 1, 8 and 40 and then with 1 mg conjugate intramuscularly on days 51, 65 and 77. Whole blood was collected from the marginal vein of the ear, clotted, the serum separated and stored at −20°.

RIA. The RIA was performed essentially as described elsewhere [21] but using a 0.01 mM citrate–phosphate buffer, pH 6,

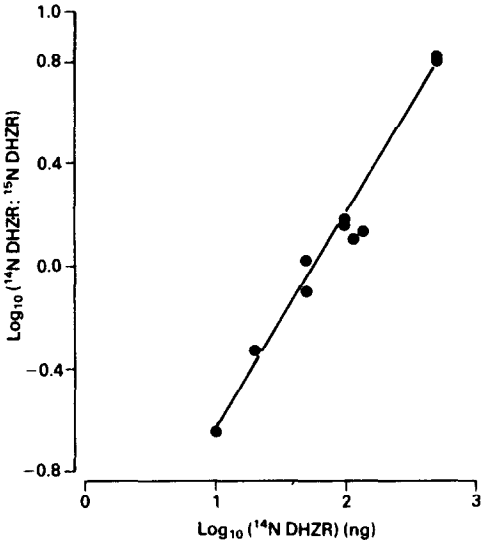

Fig. 4. GC/MS internal standard calibration line. One hundred ng of enzymically synthesized [U-^{15}N] DHZR was added to different amounts of [^{14}N] DHZR for permethylation and analysis by GC-CIMS. The ^{14}N:^{15}N ratio was calculated by monitoring the $[MH]^+$ ions at *m/z* 424 and 428 respectively.

containing 0.15 M NaCl. The assay radiotracer was synthesised from [^{3}H] DHZ (34 Ci/mmol, Amersham International) using purine nucleoside phosphorylase (E.C. 2.4.2.1, bovine spleen, Sigma Chemical Co.) after the method of [19]. [^{3}H] DHZ (10 μCi) in 100 μl 50 mM succinate buffer, pH 6.5 was warmed to 37°and 1 unit of enzyme with 100 μg ribose-1-phosphate in 100 μl succinate buffer added. The mixture was incubated overnight at 37°. The products were adjusted to pH 8 and partitioned five times against an equal vol. of H_2O satd *n*-BuOH. The *n*-BuOH fractions were combined, reduced to dryness, redissolved in 80% EtOH and the [^{3}H] DHZR purified by reverse phase HPLC.

HPLC. All chromatography was performed on columns of 5 μm Hypersil ODS (Shandon Southern Ltd) as described previously [21] except for LC/MS which is described later.

Trace enrichment was carried out after the method of [22]. Sap was centrifuged to remove debris and introduced onto the column (10 × 150 mm) via the solvent inlet. The column was then washed with H_2O (adjusted to pH 7 with triethylammonium bicarbonate) until the UV absorbance of the eluate returned to its baseline value. The cytokinins were eluted using a gradient of 0–25% MeCN in 40 min at a flow rate of 5 ml/min. [^{3}H] DHZR for use in the RIA was purified on a 10 × 150 mm column with a gradient of 8–16% MeCN in H_2O (adjusted to pH 7 as above). All other separations were performed using a gradient of 5–25% MeCN in H_2O at 5 ml/min.

Mass spectrometry. For GC/MS, samples were separated on a Flexsil OV 1701 column (12 m × 0.33 mm, coat thickness 0.6 μm; PhaseSep Ltd) equipped with an on-column injector and introduced directly into the source. Samples were injected at 300° and run at 300° using He carrier gas (1.05 kg/ml) and a detector temp of 250°. CI conditions were produced by introducing NH_3 into the source (10^{-4} torr) at 30 eV. Ions were recorded at 2 scans per sec on an Eclipse S210 computer equipped with a Kratos DS 90 operating system.

For LC/MS, samples were dissolved in 0.1 M NH_4OAc (pH 7) and chromatographed on a 4.5 × 120 mm Hypersil ODS (5 μm) column using a Gilson System 41 gradient elution system linked to the mass spectrometer and a gradient of 7–35% MeCN in 0.1 M NH_4OAc containing 8% MeCN at 1 ml/min for 20 min. The column was interfaced to the mass spectrometer via a thermospray [14,23] at a jet temp of 240° and accelerating voltage of 4 kV. Scans were recorded as above, but at 1 sec/decade, after calibration with polyethylene glycol.

Acknowledgements— This work was supported by the Agricultural and Food Research Council via a grant-in-aid to the John Innes Institute. P.G. would like to acknowledge receipt of an SERC CASE studentship. We should like to thank Fred Mellon and John Eagles, IFR, Norwich for help with the mass spectrometry and Roger Horgan for the gift of [U-^{15}N] DHZ.

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