MASS SPECTROMETRIC ANALYSIS OF CYTOKININS IN PLANT TISSUES

III. Quantitation of the cytokinin glycoside complex of lupin pods by stable isotope dilution

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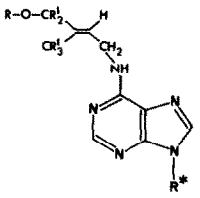
1. Introduction

O-β-D-Glucopyranosylzeatin (OGZ) and *O*-β-D-glucopyranosyl-9-β-D-ribofuranosylzeatin (OGZR), and the corresponding dihydro compounds (OGDZ and OGDZR, respectively), have been identified as metabolites of exogenously supplied zeatin in several plant tissues [1–3]. These glucosides have been isolated as naturally occurring cytokinins in plant tumour tissue [4], bean leaves [5] and sweet corn kernels [6]. In these tissues, they are presumably derived from endogenous zeatin or dihydrozeatin, or the ribosides (ZR and DZR, respectively) of these bases. However, bioassay of chromatograms of plant extracts suggests that the *O*-glucosides also occur naturally in diverse plant species (see references in [3,6]). These compounds may be important as storage forms of cytokinin and as stabilized deactivated forms of zeatin for phloem transport [3,7]. Because of the potential physiological importance of these glycosides, it is essential to develop reliable methods for their quantitation to replace bioassay procedures which lack specificity and accuracy. A mass spectrometric procedure using penta-deuterium-labelled standards is detailed herein.

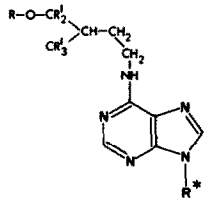
2. Materials and methods

2.1. *Extract preparation and purification*

All equipment and columns for HPLC were obtained from Waters Associates, Milford, MA. The $^2H_5$-labelled cytokinins, zeatin riboside (D_5-ZR), D_5-OGZ, D_5-OGDZ, D_5-OGZR and D_5-OGDZR, were synthe-



Metabolite	R	R'	R*
OGZ	β–D–glucopyranosyl	H	H
D_5–OGZ	β–D–glucopyranosyl	D	H
OGZR	β–D–glucopyranosyl	H	β–D–ribofuranosyl
D_5–OGZR	β–D–glucopyranosyl	D	β–D–ribofuranosyl
ZR	H	H	β–D–ribofuranosyl
D_5–ZR	H	D	β–D–ribofuranosyl



Metabolite	R	R'	R*
OGDZ	β–D–glucopyranosyl	H	H
D_5–OGDZ	β–D–glucopyranosyl	D	H
OGDZR	β–D–glucopyranosyl	H	β–D–ribofuranosyl
D_5–OGDZR	β–D–glucopyranosyl	D	β–D–ribofuranosyl
DZR	H	H	β–D–ribofuranosyl
D_5–DZR	H	D	β–D–ribofuranosyl

sized by methods detailed [8] and added to the extracting solvent used below in the amounts specified in table 1.

By procedures detailed in [8], seeds and pod walls (100 g samples) of *Lupinus luteus* (yellow lupin, plants ~14 days after petal fall) were extracted separately with 80% methanol and the two extracts were fractionated on a column of cellulose phosphate. The two eluates were subjected to preparative HPLC on Bondapak C_{18}/Porasil B (7.8 × 610 mm column) using a concave gradient of 0–100% methanol as in [8]. The following two fractions were collected, based on elution volumes previously determined with ^{2}H-labelled standards: fraction A, the volume from the end of elution of [$^2H_5$]lupinic acid to the start of D_5-ZR; fraction B, the subsequent eluate collected until the methanol concentration was 50–55%. Both fractions were evaporated, dissolved in water (1.5–2.0 ml, pH to 7) and subjected to HPLC on a μBondapak Phenyl column (3.9 × 300 mm) with a pre-column of Bondapak C_{18}/Corasil. The Phenyl column was washed with water and then eluted using a linear gradient of 0–35% methanol over 20 min. Peaks of D_5-OGZ and D_5-OGDZ were clearly evident by ultraviolet (254 and 280 nm) monitoring of the chromatogram of fraction A and these fractions were collected. Similarly fraction B yielded 3 distinct ultraviolet-absorbing fractions, one containing D_5-OGZR, one containing D_5-ZR plus D_5-OGDZR, and one with endogenous DZR (D_5-DZR was not added as a standard). The above 5 fractions from both seeds and pod walls were each run isocratically on a column (3.9 × 300 mm) of μBondapak C_{18} with a methanol–water mixture (usually 25% methanol). This step removed several unknown contaminants from the previous fractions yielding 5 fractions for mass spectral analysis from both seeds and pod walls.

2.2. *Mass spectrometric analysis*

Aliquots of the fractions purified above containing 2–5 μg of the ^{2}H-labelled cytokinins were thoroughly dried for preparation of trimethylsilyl (TMSi) derivatives. Those containing ZR, DZR, OGZR and OGDZR were derivatized at 60°C for 30 min with BSTFA–TMCS (99:1, 5 μl) and pyridine (5 μl) as solvent. The TMSi derivatives of OGZ and OGDZ were prepared by reaction at 30°C for 8 h with acetonitrile as solvent. Samples were introduced via the direct inlet probe into the mass spectrometer (DuPont 21-491B) used in the electron-impact mode. The TMSi derivatives of ZR and OGDZR were separated by differential evaporation from the probe. Spectra were recorded and stored using a VG 2025 data system. The ratios of endogenous (D_0) to $^2H_5$-labelled (D_5) cytokinins were calculated from intensities of selected ion pairs (table 1) in at least 5 scans recorded at the peak of evaporation of each compound of interest.

3. Results and discussion

In previous work, 7- and 9-β-D-glucopyranosylzeatin, zeatin and ZR were quantitated by mass spectrometry using ^{2}H-labelled internal standards [8,9]. This technique has now been further developed to permit quantitation of another important group of naturally occurring cytokinins, namely, OGZ, OGZR, OGDZ and OGDZR. The method was applied to determine the levels of these glucosides in lupin pods.

The ^{2}H-labelled internal standards added to the extracting solvent, together with any endogenous cytokinin, were recovered by fractionation on cellulose phosphate and by HPLC using in sequence Bondapak C_{18}/Porasil B, μBondapak Phenyl and μBondapak C_{18} columns. The products were converted to TMSi derivatives which were all sufficiently pure for direct-probe mass spectral analysis, a GC step being unnecessary. The ions listed in table 1 and used to calculate the ratio of endogenous (D_0) to D_5-cytokinin were selected by the following criteria:

(a) The ions were sufficiently intense in the electron-impact spectra;
(b) They contained all 5 ^{2}H atoms;
(c) They arose from fragmentations uncomplicated by others giving ions of similar *m/z* values.

Although direct-probe analysis is necessary for the TMSi derivatives of OGZR and OGDZR, which are not amenable to routine GC-MS, zeatin, ZR and OGZ, and the corresponding dihydro compounds, can be quantitated by GC-MS as TMSi derivatives. This approach was not used in the present study, which relied on direct-probe methods, but would have been useful if HPLC purification had been inadequate.

The above procedure was used to determine the levels of OGZ, OGDZ, OGZR, OGDZR and ZR in lupin pod walls and seeds. Table 1 lists amounts of D_5

Table 1
The amounts of D_5-cytokinins added to 80% methanol for extraction of lupin pods, and the ions used for mass spectral quantitation

Cytokinin	D_5-cytokinin/100 g tissue (μg)		Quantifying ions		
	Seeds	Pod walls	Origin	m/z D_0	m/z D_5
OGZ	20.0	20.0	M^+-glcO(TMSi)$_4$[a]	274	279
OGDZ	22.4	11.2	M^+-glcO(TMSi)$_4$	276	281
OGZR	20.5	20.5	M^+-glcO(TMSi)$_4$	550	555
OGDZR	19.6	19.6	M^+-glcO(TMSi)$_4$	552	557
ZR	16.0	16.0	M^+- CH_3	624	629

[a] glcO(TMSi)$_4$ denotes a tetra-TMSi glucosyloxy moiety

standards added to the solvent for extraction and identifies the fragment ions used for quantitation. Table 2 presents D_0/D_5 ratios with standard deviations and lists the levels of the above cytokinins in both seeds and pod walls. Regions of mass spectra containing the ion pairs used for quantitation in this experiment are presented in fig.1–3. The partial spectra establish that the quantitation was free from interference due to background and to complicating fragmentation. The level of DZR in the extract was also estimated indirectly.

Since developing seeds are frequently a rich source of cytokinin activity, the high levels of ZR, DZR and OGDZR observed in lupin seed (table 2) were not unexpected, the content of ZR being very similar to that found by mass spectrometry in immature sweet corn kernels [8]. However, the presence of high levels of OGDZR in lupin pod walls (table 2) was not anticipated. Although DZR was present in pod walls at 0.16 μg/g, which nearly equals that in the seeds, ZR was not present in the walls at a detectable level. In a study of cytokinins in white lupin seeds and pod walls based on bioassay methods [10] compounds with chromatographic behaviours similar to those of ZR and unidentified cytokinin glucosides were detected. In this study, such cytokinins were quantitated with unequivocal identification provided by complete mass spectra.

The results in table 1 have physiological relevance. The cytokinin content of pea seeds increases mark-

Table 2
The cytokinin content of lupin (*Lupinus luteus*) seeds and pod walls determined by mass spectrometry

Cytokinin	Seeds		Pod walls	
	D_0/D_5 ratio	Calculated level (μg/100 g)	D_0/D_5 ratio	Calculated level (μg/100 g)
OGZ	0.034 ± 0.004[a]	0.68	0.030 ± 0.005[a]	0.60
OGDZ	0.074 ± 0.004	1.7	0.72 ± 0.04	8.1
OGZR	0.18 ± 0.01	3.7	0.19 ± 0.02	3.9
OGDZR	1.97 ± 0.17	37	5.7 ± 0.9	110
ZR	1.52 ± 0.18	24	0.0	0.0
DZR[b]		20		16

[a] Standard deviation
[b] No D_5-DZR was added at extraction stage. Level was estimated from GC-MS total ion-current trace of semi-purified extracts using the ZR peak as a quantifying standard

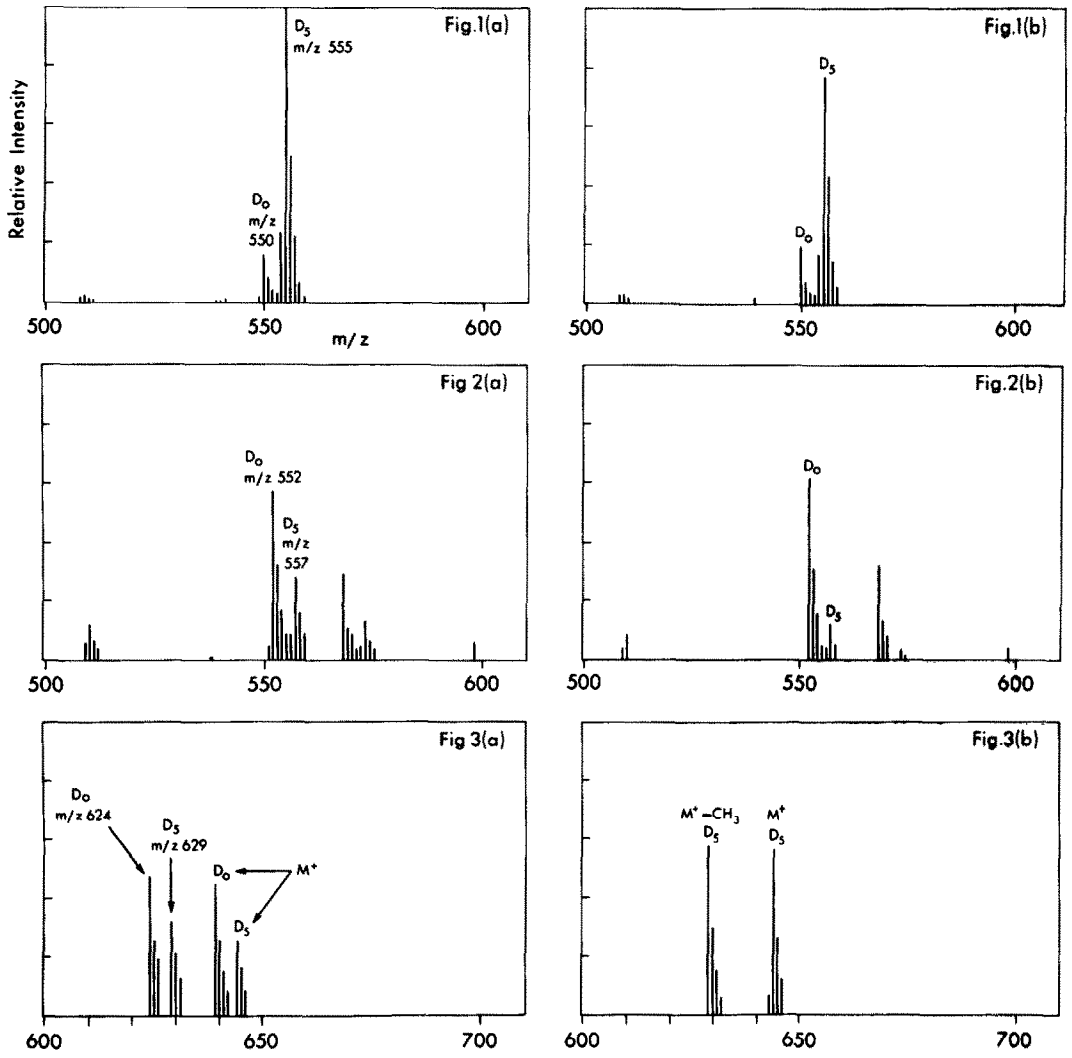


Fig.1–3. Selected regions of the mass spectra of TMSi derivatives of cytokinins purified from lupin pod extracts. The regions show ions derived from endogenous cytokinins (D_0) and $^2H_5$-labelled internal standards (D_5) and used to quantitate cytokinin levels. Fig.1. OGZR. (a) seed; (b) pod walls. Fig.2. OGDZR. (a) seed; (b) pod walls. Fig.3. ZR. (a) seed; (b) pod walls.

edly when the intact pods are cultured in vitro and this increase has been used as evidence that pea seeds synthesize cytokinins [11]. Because lupin pod walls contain DZR and a very high level of OGDZR, which is potentially capable of conversion in vivo to more active cytokinins, the reported increase in cytokinin content of pea seeds may only reflect import of cytokinins from pod walls. Hence the evidence that pea seeds synthesize cytokinins requires re-evaluation.

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