

## Identification of Cytokinin Glucosides in a Seaweed Extract

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**Abstract.** Zeatin-O-glucoside, its dihydro derivative, and dihydrozeatin riboside-O-glucoside have been identified as the main cytokinin-O-glucosides in Seasol, a commercial preparation from Tasmanian giant bull kelp marketed as a liquid organic fertilizer. The analysis, which also indicated the presence of zeatin riboside-O-glucoside, was carried out by gas chromatography–mass spectrometry of the aglucones using the stable isotope dilution method.

The use of seaweed extracts as plant growth supplements in agriculture and horticulture is common, and several of its beneficial effects are considered to be due to the presence of cytokinin like compounds (Abetz 1980, Blunden 1977). Seasol is the trade name for a seaweed extract prepared by alkaline hydrolysis of Tasmanian giant bull kelp, *Durvillea potatorum*, which is sold as a liquid organic fertilizer. Six cytokinins were recently identified and quantified in Seasol using deuterium-labeled internal standards and gas chromatography–mass spectrometry (GC-MS) (Tay et al. 1985). These were isopentenyladenine (2iP), zeatin (Z), dihydrozeatin (DZ), and their corresponding 9- $\beta$ -D-ribosides (2iPA, ZR, and DZR, respectively). This paper describes the analysis for the presence of ZOG, DZOG, ZROG, and DZROG, the side chain O-glucosides of Z, DZ, ZR, and DZR, respectively, in a partially purified Seasol extract (Fig. 1).

### Materials and Methods

#### General

Zeatin and zeatin riboside were obtained commercially (Sigma Chemical Co.). Dihydrozeatin, dihydrozeatin riboside, and the four cytokinin-O-glucosides

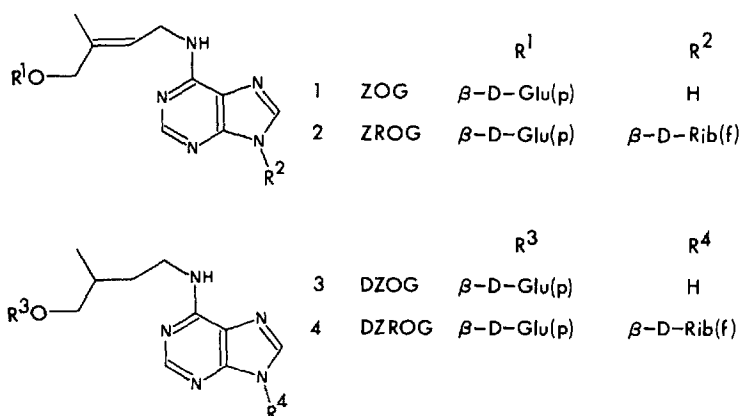


Fig. 1. Cytokinin-O-glucosides detected in Seasol.

were prepared by published methods (for references see Palni et al. 1984). The procedures for the synthesis of pentadeuterium-labeled ( $^2\text{H}_5$ ) cytokinins have also been published (Summons et al. 1979, Entsch et al. 1980).

Seasol samples were supplied by Tasbond Pty. Ltd., Tasmania. One liter of Seasol concentrate is the equivalent of  $\sim 1.5$  kg wet weight of *D. potatorum*.

#### Purification of Cytokinin Glucosides and Bioassay

Initial purification of Seasol concentrate (50 ml) was achieved by extraction with n-butanol followed by sequential chromatography on columns of insoluble polyvinylpyrrolidone and cellulose phosphate as previously reported (Tay et al. 1985). The ammonia eluate from the cellulose phosphate column, which included cytokinin bases, ribosides, and glucosides, was fractionated on a column of Sephadex LH-20 and eluted with 35% aqueous ethanol. Fractions 9–13 at the expected elution volume of cytokinin glucosides (Stuchbury et al. 1979) were pooled and dried by rotary film evaporation at 30°C. The residue was taken up in 35% ethanol (2 ml), and aliquots were taken out for soybean callus bioassay (Miller 1968). The bioassay was carried out as described by Palni et al. (1984). To the remaining sample was added 5  $\mu\text{g}$  each of  $^2\text{H}_5$ -labeled ZOG, DZOG, ZROG, and DZROG and [ $^3\text{H}$ ]-ZR (244 mCi/mM;  $\sim 300$  k DPM), which was then treated with sweet almond  $\beta$ -glucosidase (Palni and Horgan 1983). The resulting aglucones were extracted into n-butanol ( $\times 4$ ) at pH 8.1, and the butanol layers were pooled and dried. To this [ $^3\text{H}$ ]-Z (120 mCi/mM;  $\sim 18$  k DPM) was added, and the sample was rechromatographed on Sephadex LH-20 column. The two fractions containing ZR/DZR and Z/DZ were detected by radioactivity determination. Each fraction was then subjected to high-performance liquid chromatography (RCM-C<sub>8</sub> column, gradient elution with 5–50% methanol containing 0.2 M acetic acid at 3 ml/min; UV detection at  $\lambda_{254\text{ nm}}$  and  $\lambda_{280\text{ nm}}$ ) to separate the individual components—namely, ZR, DZR, and Z, DZ. These purified cytokinins were dried and further analyzed by GC-MS.

**Table 1.** Cytokinin O-glucosides in Seasol.

Cytokinin glucoside <sup>a</sup>	Derivative	Characteristic ions	m/z <sup>b</sup>	Quantity <sup>c</sup> ( $\mu\text{g/l}$ )
ZROG	TMSi	M <sup>+</sup> -TMSiOCH <sub>2</sub>	536 (539)*	<2.5 <sup>d</sup>
		M <sup>+</sup> -TMSiOH	549 (554)	
		M <sup>+</sup> -CH <sub>3</sub>	624 (629)	
		M <sup>+</sup>	639 (644)	
DZROG	TMSi	M <sup>+</sup> -TMSiOCH <sub>2</sub>	538 (541)*	13.0
		M <sup>+</sup> -TMSiOH	551 (556)	
		M <sup>+</sup> -CH <sub>3</sub>	626 (631)	
		M <sup>+</sup>	641 (646)	
ZOG	tBuDMSi	M <sup>+</sup> -C <sub>4</sub> H <sub>9</sub>	390 (395)*	15.0
		M <sup>+</sup> -CH <sub>3</sub>	432 (437)	
		M <sup>+</sup>	447 (452)	
DZOG	tBuDMSi	M <sup>+</sup> -C <sub>4</sub> H <sub>9</sub>	392 (397)*	22.0
		M <sup>+</sup> -CH <sub>3</sub>	434 (439)	
		M <sup>+</sup>	449 (454)	

<sup>a</sup> Cytokinin glucosides were determined as their respective aglucones.

<sup>b</sup> The ions in parentheses are derived from the deuterium-labeled cytokinin-O-glucosides added as internal standards before  $\beta$ -glucosidase treatment.

<sup>c</sup> Values represent minimum levels and are expressed as  $\mu\text{g/l}$  of Seasol concentrate based on the ion pair marked with an asterisk. Values have been corrected for partial deuteration of some of the internal standards used.

<sup>d</sup> Approaching detection limit of instrumental method.

The recoveries of ZR and Z after HPLC were 55% and 80%, respectively, based on radioactivity levels.

### Gas Chromatography–Mass Spectrometry

Trimethylsilyl (TMSi) derivatives of ZR and DZR and tert.-butyldimethylsilyl (t-BuDMSi) derivatives of Z and DZ were prepared as previously reported (Tay et al. 1985, Palni et al. 1985). GC-MS was carried out under electron impact (EI; 70 eV) conditions on a Finnigan 4510 instrument interfaced to an IncoS data system using a capillary column (BP5, 25 m  $\times$  0.33 mm, 1- $\mu\text{m}$ -thick stationary phase; He carrier gas; 250°C (for 2 min) to 280°C at 10°C/min). Partial mass spectra were obtained by scanning over a selected mass range (m/z 530–661 for ribosides and m/z 385–460 for bases). The ions used for identification and quantitation are listed in Table 1.

### Results and Discussion

In an earlier paper, the identification and quantitation of several cytokinin bases and ribosides present in a seaweed extract (Seasol) were described (Tay et al. 1985). Because cytokinin glucosides had not been reported as occurring in lower plants, the analysis was not originally designed to measure levels of

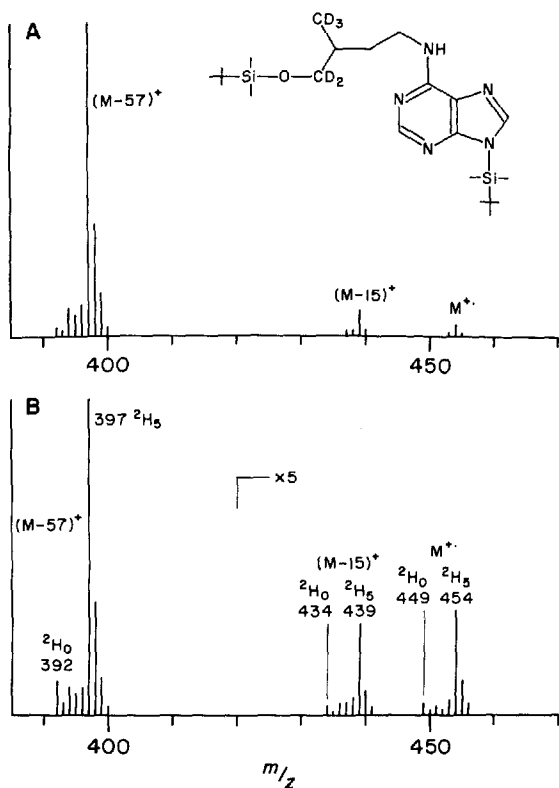


Fig. 2. Electron impact GC mass spectra of the molecular ion regions of (A) standard (tBuDMSi)<sub>2</sub>-[<sup>2</sup>H<sub>5</sub>]-DZ, and (B) (tBuDMSi)<sub>2</sub>-DZ fraction resulting from enzymatic hydrolysis of DZOG. <sup>2</sup>H<sub>5</sub> and <sup>2</sup>H<sub>0</sub> ions are from the added internal standard and endogenous cytokinin, respectively.

these compounds in the extract. In the event, the elution volume from LH-20 chromatography corresponding to the "glucoside fraction" was tested for cytokinin activity using the soybean callus bioassay and gave a dose-dependent positive response. This indicated the presence of cytokinin-O-glucosides in the sample, since cytokinin conjugates in which the glucosyl moiety is attached directly to the purine nucleus (the N-glucosides) are essentially inactive in the soybean callus test (Palni et al. 1984). After the addition of <sup>2</sup>H<sub>5</sub>-cytokinin-O-glucosides (ZOG, DZOG, ZROG, and DZROG) as internal standards, the remainder of the glucoside fraction was treated with β-glucosidase, and the resulting aglucones were purified by chromatography on Sephadex LH-20 followed by HPLC, using [<sup>3</sup>H]-Z and ZR to detect the eluting cytokinin fractions.

The individual cytokinins from HPLC were derivatized, and the presence of endogenous Z, DZ, ZR, and DZR was determined by GC-MS (Fig. 2). The amounts of these compounds in the partially purified "glucoside fraction" from Sephadex LH-20 chromatography were measured by comparison of the specific ion responses of the endogenous (<sup>2</sup>H<sub>0</sub>) cytokinins and their corresponding added <sup>2</sup>H<sub>5</sub>-labeled internal standards (Fig. 3, Table 1).

Zeatin riboside and its dihydro derivative were analyzed as their TMSi derivatives, whereas t-BuDMSi derivatives were preferred for Z and DZ. The

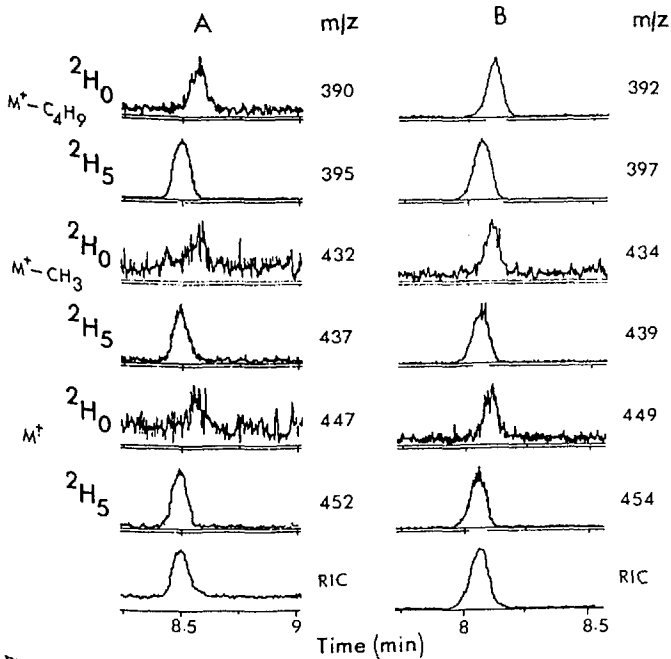


Fig. 3. Ion chromatograms from partial GC-MS scans of (A)  $(t\text{BuDMSi})_2\text{-Z}$ , and (B)  $(t\text{BuDMSi})_2\text{-DZ}$  fractions purified from the  $\beta$ -glucosidase-treated "glucoside fraction" following Sephadex LH-20 chromatography.

value of  $t\text{-BuDMSi}$  derivatives for the GC-MS analysis of cytokinin bases has been previously discussed (Palni et al. 1985). It can be seen from the mass spectrum of  $(t\text{BuDMSi})_2\text{-}[^2\text{H}_5]\text{-DZ}$  (Fig. 2A) that the fragment ion  $M^+-57$  is considerably more intense than the molecular ion ( $M^+$ ) or  $M^+-15$  ion and has been used for quantitation in the present study (Fig. 2B, Table 1). These endogenous cytokinins could only have resulted from the enzymatic hydrolysis of the respective O-glucosides, since cytokinin-N-glucosides (the 7- and 9- glucosides), which coelute with the O-glucosides on Sephadex LH-20 column, are resistant to  $\beta$ -glucosidase treatment and could not have contributed to the aglucones detected in this study. There has also been a recent report of the presence in *Pinus radiata* buds of a hexoside of ZR in which the hexose moiety is attached to the ribosyl group (Taylor et al. 1984). The complete structure of this ZR metabolite has not been established, however, and there is no evidence that it is hydrolyzed by  $\beta$ -glucosidase.

The values given in Table 1 for the O-glucosides are minimum ones, since the  $^2\text{H}_5$  internal standards were added after several purification steps had been carried out, and thus the GC-MS measurements would not take into account any losses occurring up to the point of addition. These losses could be particularly substantial for the O-glucosides of ZR and DZR. For example, the reported partition coefficient (0.27) of ZROG in  $n\text{-butanol/water}$  (Scott et al.

1982) means that at least 50% of this cytokinin would have been lost during the initial n-butanol extraction step. Even allowing for the fact that measurements were carried out on a partially purified extract, three of the four O-glucosides—ZOG, DZOG, and DZROG—were present in substantial amounts, whereas the fourth, ZROG, was barely above the minimum detection level of the instrumental method. Its identification in the seaweed extract must be regarded as tentative, particularly since the added [ $^3\text{H}$ ]-ZR would also have contributed marginally to the [ $^2\text{H}_0$ ]-ZR response. In recent years, O-glucosides of cytokinins have been identified conclusively in a number of plant tissues and appear to be ubiquitous cytokinins in higher plants (Letham and Palni 1983). The detection of cytokinin-O-glucosides in *Seasol* described here is the first definitive report of their occurrence in an extract obtained from a lower plant.

Cytokinins can produce some of the beneficial effects claimed for seaweed extracts, and it has been suggested that these compounds may be responsible for much of the activity of seaweed extracts (Brain et al. 1973). The observed cytokinin levels (Table 1; Tay et al. 1985) do not, however, seem sufficiently high to indicate that these are the only active components in *Seasol* responsible for its reported effects on plants, particularly in view of the high dilutions of *Seasol* used in practice.

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