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DETECTION OF CYTOKININS IN A SEAWEED EXTRACT

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Key Word Index—*Durvillea potatorum*; Tasmanian Giant Bull kelp; Seasol; cytokinins; GC/MS; stable isotope dilution; bioassay.

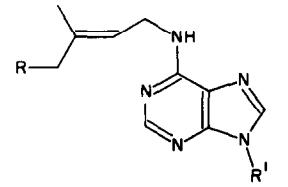
Abstract—*trans*-Zeatin, *trans*-zeatin riboside, their dihydro derivatives, isopentenyladenine and isopentenyladenosine have been identified and quantified in Seasol, a commercial extract of Tasmanian Giant Bull kelp, *Durvillea potatorum*.

INTRODUCTION

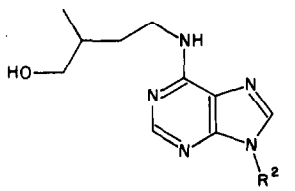
Numerous claims have been made of the beneficial effects of the use of seaweed extracts in agriculture and horticulture. A recent review [1] lists literature reports which attest to the efficacy of seaweed extracts in promoting higher crop yields, increasing the resistance of plants to frost, fungal diseases and insect attack, reducing storage losses of fruits, increasing nutrient absorption and improving livestock health from grazing on treated crops and pastures. Many of these effects have been attributed to the presence of cytokinins in seaweed extracts. Bioassay results have indicated the presence of cytokinin activity in seaweed preparations, although these show wide variations even between batches of the same product [2].

Seasol is the trade name of one of a number of commercially available seaweed extracts. It is prepared by alkaline hydrolysis of Tasmanian Giant Bull kelp, *Durvillea potatorum*, and is marketed as a 'liquid organic seaweed' for use as a plant food and soil conditioner.

In this paper we present data on the identification and quantification of several cytokinins in Seasol using a mass spectrometric stable isotope dilution method [3–5].



	R	R^1
1	OH	H
2	OH	β-D-ribosyl
3	H	H
4	H	β-D-ribosyl



	R^2
5	H
6	β-D-ribosyl

RESULTS AND DISCUSSION

Seasol is commercially produced by alkaline hydrolysis of *D. potatorum* at elevated temperature and pressure, and contains a large number of degradation products and additives. This necessitated extensive sample purification (see Experimental) before a satisfactory cytokinin analysis could be achieved. In all, six cytokinins, *trans*-zeatin, **1** (Z), *trans*-zeatin-9-β-D-riboside, **2** (ZR), their dihydro derivatives, **5** (DZ) and **6** (DZR), N^6-(Δ^2-isopentenyl)adenine, **3** (2iP) and its 9-β-D-riboside, **4** (2iPA), were identified and quantified by GC/MS in the multiple ion detection mode (GC/MS-MID) (Table 1). Addition of [^{3}H]ZR to seaweed extracts facilitated detection of the 'total cytokinin fraction' during the early stages of extraction and chromatography, as well as giving a measure of the percentage recovery of ZR throughout the purification procedure (Table 2). Although sample purification for the soybean callus bioassay was kept to a minimum, a bulk HPLC step to obtain a 'total cytokinin fraction' was considered desirable to remove impurities which could interfere with the assay. No appreciable differences were observed in the soybean callus bioassay response for the individual extracts using authentic ZR for reference. However, increased callus yield at higher sample concentrations for the three samples indicate that the purification procedure used was adequate to remove possible interfering substances from the seaweed extracts. In addition, these results clearly suggest the presence of cytokinin-like compounds in the extracts. Quantitative extrapolation from the bioassay data may not, however, be valid for a variety of reasons [6, 7]. Qualitatively the bioassay results do support the findings of the GC/MS-MID analysis carried out on Seasol.

Four deuterium labelled cytokinin analogues of Z, ZR, 2iP and 2iPA, together with [^{3}H]ZR, were added to seaweed extract 2 (marketed as Seasol) prior to initial extraction and chromatography. As well as permitting quantitative mass spectrometric analysis of any corresponding cytokinins present in the extract, the deuterated analogues can act as 'carriers' for the small quantities of endogenous cytokinins likely to be present [8]. Separation of the 'total cytokinin fraction' from bulk impurities in the seaweed extract was by sequential CC steps on polyvinyl pyrrolidone (PVP) and cellulose phosphate (CP) and monitored by radiotracer counting of

Table 1. Quantification of cytokinins in Seasol

Cytokinin derivative	Ions monitored by MID		Quantity (μg/l.)*
	$[M]^+$	$[M-Me]^+$	
TMSi-*trans*-ZR	639 (644)	624 (629)	7.01 ± 1.0
TMSi-2iPA	551 (557)	536 (542)	2.06 ± 1.04
TMSi-DZR	641 (646)	626 (631)	36.59 ± 3.18
t-BuDMSi-2iP	317 (319)	302 (304)	15.94 ± 1.5
	$[M]^+$	$[M-OMe]^+$	
Permethyl-*trans*-Z	261 (266)	230 (235)	0.70 ± 0.3
Permethyl-DZ	263 (265)	232 (234)	1.06 ± 0.02

*Values are expressed as μg/l. of concentrate, based on the more intense ($[M-Me]^+$ or $[M-OMe]^+$) ions and are the mean of three determinations. The values in parentheses are of ions from the deuterated cytokinins added as internal standards.

Table 2. Recovery of [^{3}H]ZR from the purification procedure*

	n-BuOH	PVP	CP	LH-20	HPLC
Bioassay samples					
Seaweed sample 1	92	89	74	—	61
Seaweed sample 2	92	92	79	—	61
Seaweed sample 3	92	86	70	—	55
GC/MS-MID sample					
Seaweed sample 2	92	90	80	80	53

*Expressed as a percentage of initially added [^{3}H]ZR.

[^{3}H]ZR (Table 2). Preliminary separation of individual cytokinins in the 'total cytokinin fraction' was on Sephadex LH-20 and was facilitated by prior addition of [^{3}H]2iP.

Four fractions A–D (see Experimental) were collected from the Sephadex LH-20 column. Fraction A contained [^{3}H]ZR together with [$^2H_5$]ZR and any endogenous ZR, *cis*-ZR and DZR. To this fraction was added [$^2H_5$]DZR (5 μg) to quantify the level of any DZR which might be present. Fraction B contained [$^2H_5$]Z plus any endogenous Z, *cis*-Z and DZ. To this, [$^2H_2$]DZ (5 μg) and [^{3}H]Z were added. To fraction C, containing [$^2H_6$]2iPA and any 2iPA present, [^{3}H]2iP was added. Fraction D contained [$^2H_2$]2iP, [^{3}H]2iP and any endogenous 2iP. All radioactive markers used were of high specific activity and were added in amounts well below the GC/MS-MID detection level. It was necessary to add the radioactive markers to each fraction to detect the cytokinin bands during HPLC of the individual fractions A–D since (i) their UV absorption was often obscured by the other strongly absorbing components and (ii) HPLC R_t data, obtained using authentic cytokinins, was not effective in locating cytokinin peaks in a multicomponent system.

In the case of fraction A, two HPLC steps were carried out in order to obtain relatively pure cytokinin peaks. Figure 1(a) shows the initial HPLC run, using solvent system A, in which the radiotracer profile for the ZR/DZR band is shown superimposed on the UV trace. Following a second HPLC step (system B), bands due to ZR and DZR could be separated (Fig. 1b) and were collected for GC/MS analysis. Any *cis*-ZR present co-eluted with DZR in this solvent system. A single HPLC step was sufficient to purify the other fractions, B–D. The Z and DZ (with any co-eluting *cis*-Z) were recovered from fraction B using solvent system C; 2iPA and 2iP were purified from fractions C and D, respectively, with solvent system D. HPLC analysis of fraction C is shown in Fig. 1(c).

The cytokinin fractions purified by HPLC were derivatized and analysed by GC/MS-MID. Different derivatives [TMSi, permethyl, t-butyldimethylsilyl (t-BuDMSi)] were evaluated using authentic standards on a sub-microgram scale, to optimize sensitivity and selectivity for individual cytokinins. For each derivative, a standard curve was produced by plotting the observed intensity ratios of the ions monitored against the known molar ratio for mixtures of unlabelled and labelled cytokinin standards [9]. The three ribosides, ZR, DZR and 2iPA, were analysed as their per-TMSi derivatives [10], whereas the TMSi derivatives of the cytokinin bases Z, DZ and 2iP gave non-reproducible GC responses. The t-BuDMSi derivative [11–13] was found to be the derivative of choice for 2iP. Using the same conditions, both Z and DZ gave mixtures of the mono- and di-t-BuDMSi derivatives and were, therefore, analysed as their permethyl derivatives [14–16]. More recently, it has been found that inclusion of a catalyst (4-dimethylaminopyridine, DMAP) in the t-BuDMSi derivatization mixture overcomes the problem of formation of two derivatives for Z and DZ, and produces only the di-t-BuDMSi compounds [17, 18].

For improved selectivity, four high mass ions were

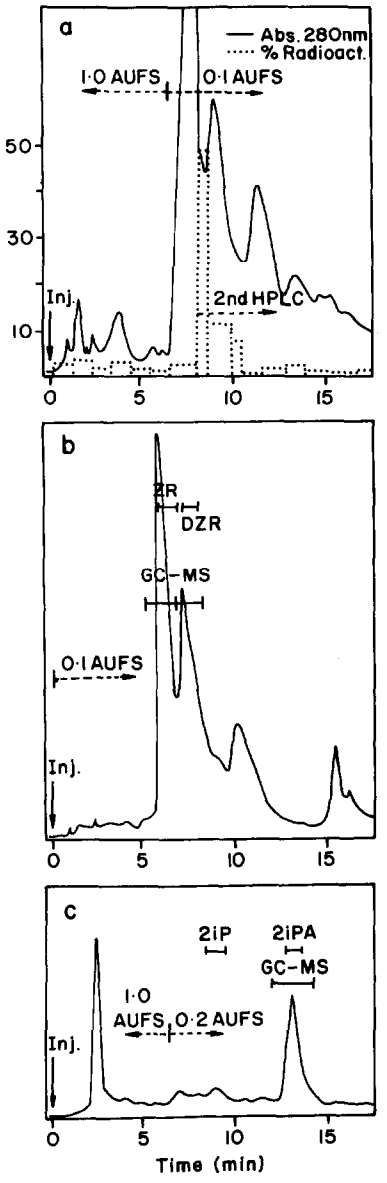


Fig. 1. Reversed-phase HPLC UV traces for Sephadex LH-20 fraction A (a and b) and fraction C (c) of Seasol seaweed extract sample. For details of solvent systems see Experimental.

monitored for each derivatized cytokinin fraction in the GC/MS-MID analyses. These were the $[M]^+$ and $[M-Me]^+$ ions (TMSi and t-BuDMSi) or $[M]^+$ and $[M-OMe]^+$ ions (permethyl) for the deuterium labelled and unlabelled (i.e. endogenous) derivatives (Table 1). Quantification was based on the fragment ions which were more intense than the $[M]^+$. Allowance was made for incomplete deuterium incorporation in the labelled standards (e.g. $[^2H_5]$DZR contained 63.1% $^2H_5$). In addition, individual mass spectral scans (m/z 400–650) were obtained for derivatized ZR and DZR fractions, which confirmed the absence of any other components which might fortuitously have had the same GC R_t and produce the same ions as those selected for monitoring. The six cytokinins, **1–6**, have all been identified and quantified in Seasol. It should, however, be noted that the values obtained for DZ and DZR are underestimates as the corresponding deuterated analogues were added to the appropriate fractions only after Sephadex LH-20 CC. Losses prior to that step are, therefore, not accounted for. Under the GC conditions used, any endogenous *cis*-ZR co-eluting with the DZR fraction from HPLC could be separated. Within the detection limits of the GC/MS-MID system, no *cis*-ZR was found. Similarly, no endogenous *cis*-Z was detected by GC/MS-MID in the DZ fraction from HPLC.

The occurrence of cytokinins in a seaweed extract described here constitutes the first definitive report on the identification of cytokinins from algae. The observed levels (Table 1) do not, however, appear to be sufficiently high to suggest that these are the only compounds responsible for the reported beneficial effects of Seasol on plants (R. Bayer, personal communication). Cytokinin activity was also detected by Soybean callus bioassay in early fractions eluting from Sephadex LH-20. Work is in progress to identify the cytokinin-active compound(s) in these fractions which correspond to the elution volume expected for cytokinin glucosides. The presence in a seaweed extract of Z, ZR and high levels of their dihydro derivatives, cytokinins normally associated with higher plants, is unusual. The absence of any detectable levels of *cis*-isomers of Z and ZR in the extract suggests that breakdown of *t*-RNA under the hydrolysis conditions is not responsible for cytokinins **1–6** in Seasol.

EXPERIMENTAL

General. Solvents used were of AR grade and were redistilled before use. Zeatin (Z, **1**), isopentenyladenine (2iP, **3**) and their corresponding 9-β-D-ribosides (ZR, **2**, and 2iPA, **4**, respectively) were obtained commercially. Dihydrozeatin (DZ, **5**) and its 9-β-D-riboside (DZR, **6**) and all other cytokinin standards were prepared by published methods (for refs see ref. [19]). The procedures for the synthesis of corresponding ^2H-labelled compounds ($[^2H_5]$Z, $[^2H_5]$ZR, and $[^2H_5]$DZR, $[^2H_2]$DZ, $[^2H_2]$2iP and $[^2H_6]$2iPA) have been reported earlier [4, 20]. ^3H-labelled cytokinins were obtained by heating the respective non-radioactive compounds with ^3H_2O [21].

Seaweed extracts. Samples 1–3 were kindly supplied by Tasbond Pty. Ltd., Tasmania, as extracts of Tasmanian Giant Bull kelp. Samples 1 and 2 were prepared by alkaline hydrolysis at elevated temp. and pres. Seaweed extract 1 (pH 11) was without added stabilizing agents and pH adjusters while, to sample 2 (pH 9.6, marketed as Seasol), urea and phosphoric acid had been added. Sample 3 was made by subjecting seaweed to high speed dispersion in H_2O at an elevated temp. (*ca* 130°) and pres (28 psi) for 7 hr. In 1 l. Seasol concentrate is the equivalent of *ca* 1.5 kg wet wt of *D. potatorum*.

Purification of cytokinins. Seasol concentrate (50 ml) was dissolved in H_2O (500 ml) and ^2H-labelled cytokinins were added (10 μg each of $[^2H_5]$ZR, $[^2H_5]$Z, $[^2H_6]$2iPA and $[^2H_2]$2iP) as int. standards for quantification. At the same time $[^3H]$ZR (370000 dpm) was also added. Sample pH was adjusted to 8.2 with 1 M NaOH and extracted × 3 with an equal vol. of H_2O satd *n*-BuOH. The *n*-BuOH layers were pooled, dried by rotary film evaporation at 30° and the extract then subjected to chromatography on insoluble PVP (pH 3.5) followed by cation exchange chromatography on cellulose phosphate (CP) (NH_4^+ form, pH 3.1) as in ref. [19]. To the NH_3 eluate, which included cytokinin bases and glycosides, $[^3H]$2iP (500000 dpm) was added. The sample was then evaporated to dryness, dissolved in

35% aq. EtOH (2.5 ml) and fractionated on a column of Sephadex LH-20 (bead size 25–100 μm, 80 × 2.5 cm) eluting with the same solvent at a descending flow rate of 30 ml/hr [19]. Fifty 1-hr fractions were collected and an aliquot from each was taken for liquid scintillation counting. This allowed detection of fractions containing [^{3}H]ZR and [^{3}H]2iP. Appropriate fractions were pooled for further analysis as follows: fractions 14–16 for analysis of ZR and DZR (fraction A); fractions 18–20 for analysis of Z and DZ (fraction B); fractions 22–26 for analysis of 2iPA (fraction C); fractions 28–32 for analysis of 2iP (fraction D). In addition fractions 9–13 were also combined since cytokinin glucosides would be expected to elute in this region [22].

For bioassay expts, an aliquot (15 ml) from each of the three seaweed extract samples 1–3, were diluted with H_2O (100 ml) and, to each, [^{3}H]ZR (350 000 dpm) was added. Each was then subjected to a *n*-BuOH partition step, followed by CC on PVP and CP as above. The cytokinins in the NH_3 eluates following CP chromatography were concd by a bulk HPLC step and then used directly for determination of the total cytokinin activity using the soybean callus test.

Bioassay. Glycine max L. Merr. cv Acme callus was maintained on basal medium [23] solidified with agar (0.8%, w/v) and supplemented with α-naphthaleneacetic acid (NAA) (2 mg/l.) and 6-benzylaminopurine (1 mg/l.) at 26° in the dark. Cytokinin bioassays were performed in duplicate in 50 ml Erlenmeyer flasks to which 25 ml of medium containing test samples had been added. Three callus explants were added to each flask and the total callus yield was determined after 4 weeks [24].

HPLC. The equipment has been described in detail elsewhere [25]. Solvents used were mixtures of MeOH and H_2O containing 1% HOAc, the proportion of MeOH being expressed as a percentage by vol. A flow rate of 3 ml/min was used (except 1 ml/min for bulk HPLC) and the column eluate was monitored by its *A* at 280 nm. The details of column/solvent combinations used in the present study were as follows: C_8 radial compression column (8 × 100 mm) eluted with a linear gradient of 5–45% MeOH in 30 min (system A), or isocratic elution with 15% MeOH (system B); μBondapak C_{18} column (7.8 × 300 mm) eluted with a linear gradient of 10–30% MeOH in 30 min (system C), or isocratic elution with 35% MeOH (system D). For the preparation of samples for bioassay the μBondapak C_{18} column was used, the column being washed with 10% MeOH for 40 min after sample loading and the 'bulk cytokinin fraction' was then eluted with 70% MeOH.

GC/MS. TMSi derivatives were prepared by heating the sample with 10 μl pyridine–*N*-methyl-*N*-TMSi-trifluoroacetamide (MSTFA) (1:1) at 90° for 10 min. For the preparation of t-BuDMSi derivatives, *N*-methyl-*N*-t-butyldimethylsilyl trifluoroacetamide (MTBSTFA, containing 1% *trans*-BuDMCSi) was used instead of MSTFA, under the same conditions [18]. Permethylated cytokinins were made by a modified Hakomori method [26]. The dried permethylated cytokinin samples were dissolved in EtOAc (10 μl) for GC/MS. GC was carried out using a 1.5 m × 2 mm i.d. glass column, 3% OV-101 on 100–200 mesh Gas-chrom Q, He flow rate 25 ml/min; temp. programme 200° for 2 min then to 300° at 10°/min. EIMS: 70 eV; source, separator and inlet line temp. 200°, 280° and 250°, respectively. MID was carried out by computer controlled voltage switching between the ions given in Table 1, ion sampling time 0.2 sec, mass window ±0.4 mass units.

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