Phytochemistry, Vol. 28, No. 2, pp. 337–338, 1989.
Printed in Great Britain.

0031-9422/89 $3.00 + 0.00
Pergamon Press plc.

IDENTIFICATION OF A CYTOKININ IN THE GREEN ALGA *CHARA GLOBULARIS*

WEI ZHANG, HISAKAZU YAMANE,* NOBUTAKA TAKAHASHI,* DAVID J. CHAPMAN and BERNARD O. PHINNEY

Department of Biology, University of California at Los Angeles, Los Angeles, CA 90024, U.S.A.; *Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo, Japan

(*Received in revised form* 12 *April* 1988)

Key Word Index—*Chara globularis*; Characeae; GC/MS; cytokinin; isopentenyladenosine.

Abstract—Isopentenyladenosine was chemically identified from the green alga *Chara globularis* by combined GC/MS. This is the first chemical identification of a cytokinin in green algae.

INTRODUCTION

The phytohormones which are widespread in higher plants, have been identified unequivocally in a few instances only in algae. Auxins (IAA) have been found in the brown alga *Undaria pinnatifida* [1] and the green alga *Caulerpa paspaloides* [2], while the green alga *Stigeoclonium* sp. has been shown to produce abscisic acid [3]. Phenylacetic acid has been identified in *Undaria pinnatifida* [4] and *Enteromorpha compressa* [5]. Recently, a new class of plant hormones, the Brassinosteroids, which promote growth of higher plants, have been found in an alga [6].

For cytokinins, known to be ubiquitous amongst higher plants (Angiospermae), there are relatively few reports suggesting their presence in algae. Indirect evidence comes from bioassays and co-chromatography of algal cytokinin-like substances with known cytokinins [7]. The only definitive evidence for the identification of cytokinins from algae is by GC/MS, using the algal preparation 'seasol', a commercially available industrial product obtained by alkaline hydrolysis of the brown alga *Durvillea potatorum* [8, 9].

Similar techniques have been used to identify a cytokinin in seawater from the *Fucus-Ascophyllum* zone [10], although the presence of cytokinins in those brown algae has not been established. Presumptive evidence (HPLC) has been provided for cytokinins in the brown alga *Ecklonia maxima* [11].

Chara globularis belongs to the order to Charales in the class of Charophyceae, a group generally considered to be the ancestor of land plants. In this paper we report the definitive, chemical identification of the cytokinin, isopentenyladenosine from this alga.

RESULTS AND DISCUSSION

The ammonium hydroxide eluate of the neutral ethyl acetate fraction from a P-1 cellulose phosphate column gave a negative bioassay result, suggesting the absence of cytokinins in this fraction.

In contrast, the ammonium hydroxide eluate of the aqueous fraction from the P-1 cellulose phosphate column was bioactive. The eluates from the next purification on a Sephadex LH-20 column ($V_e/V_t = 1.45$–1.64) showed bioactivity. After further purification by HPLC, the fractions with R_ts 38–40 min) similar to that of authentic isopentenyladenosine, showed bioactivity.

When analysed with GC/MS, the total ion current peak of the trimethylsilyl derivatives of these cytokinin-active fractions from HPLC showed the same R_t (10′ 56″) as that of the authentic TMSi-isopentenyladenosine (10′ 55″). The two peaks gave identical mass spectra (Table 1). Thus the cytokinin in *Chara globularis* was chemically identified as isopentenyladenosine. The amount of the cytokinin detected at the GC/MS stage was *ca* 0.4 μg/kg fresh weight algae, based upon a comparison with a known amount of authentic sample.

Isopentenyladenosine may be produced either by *de novo* synthesis or from degradation or turnover of cytokinin-containing tRNA. This work does not distinguish between either of these three possibilities.

Although in this experiment only one kind of cytokinin was detected, this does not mean that there are no other species of cytokinins in *C. globularis*. They may be present at low levels and therefore not detectable with present instrumentation. While zeatin and its derivatives are usually the main cytokinins identified in flowering plants [12, 13], isopentenyladenine and its derivatives appear to be the main cytokinins in lower green plants. Isopentenyl-

Table 1. Comparison of MS data of authentic isopentenyladenosine and isopentenyladenosine isolated from *Chara globularis*

Sample	Principal ions and their relative intensities (% base peak)
Authentic $(TMSi)_3$-isopentenyladenosine	551 (M^+, 20), 536 (11), 508 (5), 259 (11), 245 (20), 232 (40), 230 (29), 203 (29), 73 (100)
$(TMSi)_3$-isopentenyladenosine from *C. globularis*	551 (M^+, 20), 536 (9), 508 (5), 259 (8), 245 (15), 232 (37), 230 (28), 203 (28), 73 (100)

adenine and derivatives have been identified as the principal cytokinins in the moss *Physcomitrella* (mutants [14] and callus hybrids [15]). They also seemed to be the main cytokinins in algae as judged by the results of bioassays and chromatography [10, 11]. The identification of isopentenyladenosine in *C. globularis* adds to the list of lower green plants producing isopentenyladenine and derivatives as the principal cytokinins, and is the first identification of cytokinins in green algae.

EXPERIMENTAL

Plant material. Chara globularis at the vegetative stage was collected from Tujunga creek in Big Tujunga Canyon, Los Angeles County, California. The alga was cleaned, rinsed and stored at −20° until used.

Extraction and solvent fractionation. All the solvents used were reagent grade and were redistilled before use. The whole algal thalli (5 kg) were homogenized and twice extracted at 4° for 2 days with cold 80% aq. MeOH. The extracts were filtered, pooled and concd under red. pres. at 30° to an aq. residue (800 ml). The aq. residue was then partioned with *n*-hexane (3 × 600 ml), and the aq. phase was adjusted to pH 3 with 6 M HCl and re-extracted with EtOAc (4 × 600 ml) to give an aq. fraction and an EtOAc fraction. The EtOAc fraction was partitioned with 5% aq. $NaHCO_3$, passed through a dry Na_2SO_4 column, and evapd to dryness under red. pres. at 30° to give a neutral EtOAc fraction.

Purification. The aq. fraction was adjusted to pH 6 with conc NH_4OH and extracted with H_2O satd *n*-BuOH (3 × 600 ml). The *n*-BuOH fractions were pooled and evapd to dryness at 40° under red. pres. The residue was redissolved in 0.5 M aq. HOAc (5 ml) and chromatographed with 500 ml 0.05 M aq. HOAc using a PVPP column (22 × 200 mm bed vol.) at an elution rate of 1.5 ml/min. The eluate was then adjusted to pH 3 with 6 M HCl and subjected to cation exchange chromatography on a P-1 cellulose phosphate (NH_4^+ form) column (55 × 250 mm bed vol.), eluted with 0.05 M HOAc (800 ml), H_2O (500 ml), and then 2 M aq. NH_4OH (1500 ml) with an elution rate of 2 ml/min. The NH_4OH eluate was concd to 300 ml under red. pres. at 40°, and extracted with H_2O satd *n*-BuOH (3 × 100 ml). The *n*-BuOH fractions were pooled and evapd to dryness under red. pres. at 40°. The residue was dissolved in 35% aq. EtOH (1 ml) and chromatographed on a Sephadex LH-20 column (22 × 450 mm bed vol.), eluting with 35% aq. EtOH at a rate of 0.7 ml/min. 33 30 ml fractions were collected and each was subjected to *Amaranthus* seed bioassay (see below). The cytokinin active fractions were pooled and further purified by HPLC with a 5μ Nucleosil ODS column (10 × 150 mm). Following injection, the column was eluted isocratically with 25% MeOH in 1% aq. HOAc for the first 15 min, followed by a 15 min linear gradient from 25 to 60% MeOH in 1% aq. HOAc, and finally isocratically with 60% MeOH in 1% aq. HOAc. The flow-rate was 1.5 ml/min with UV detection at 255 nm. Each fraction was bioassayed and the active fractions pooled and repurified on HPLC as described.

The neutral EtOAc fraction was dissolved in $CHCl_3$ (100 ml) and extracted with pH 3 dilute HCl (3 × 150 ml). The aq. fractions were pooled, adjusted to pH 8 with conc NH_4OH and extracted with H_2O satd *n*-BuOH (3 × 50 ml). The *n*-BuOH extracts were pooled and evaporated under reduced pressure at 40°. The residue was redissolved in 0.5 M aq. HOAc (5 ml) and purified by PVPP chromatography, followed by P-1 cellulose phosphate chromatography as described for the aq. fraction.

Bioassay. Cytokinin-like substances were detected by the *Amaranthus* betacyanin assay, following the method of ref. [16]. For each bioassay a sample equivalent to 300 g fr. wt of alga was used.

GC/MS. GC/MS was carried out on a JEOL DX-303 with an ionization voltage of 70 eV. The sample was trimethylsilylated with pyridine-*N*-methyl-*N*-TMSi-trifluoroacetamide (MSTFA) at 80° for 30 min and injected onto a fused silica chemically bound capillary column DB-1 (15 m × 0.258 mm i.d., 0.25 μm thickness) at 160° in the splitless mode. After 2 min isothermal hold (sampling time), the column temp was raised 16° per min to 260°, followed by a 10 min isothermal hold. The pressure of the He carrier gas was 0.6 kgf/cm. The column led directly into the ion source.

Acknowledgements—We would like to thank Mr M. Nakayama (Department of Agricultural Chemistry, University of Tokyo, Japan) for GC/MS work and Drs C. Spray and S. Fujioka, (Department of Biology, UCLA, U.S.A.) and Dr T. Yokota, (Department of Agricultural Chemistry, University of Tokyo, Japan) for useful discussion. This work was supported by NSF grant (BSR-14838) to David J. Chapman.

REFERENCES

1. Abe, H., Uchiyama, M. and Sato, R. (1972) *Agric. Biol. Chem.* **36**, 2259.
2. Jacobs, W. P., Falkenstein, K. and Hamilton, R. H. (1985) *Plant Physiol.* **78**, 844.
3. Tietz, A. and Kasprik, W. (1986) *Biochem. Physiol. Pflanzen.* **181**, 269.
4. Abe, H., Uchiyama, M. and Sato, R. (1974) *Agric. Biol. Chem.* **38**, 897.
5. Fries, L. and Aberg, S. (1978) *Z. Pflanzenphysiol.* **88**, 383.
6. Yokota, T., Kim, S. M., Fukui, Y., Takahashi, N., Takeuchi, Y. and Takematsu, T. (1987) *Phytochemistry* **26**, 503.
7. Mooney, P. A. and Van Staden, J. (1986) *J. Plant Physiol.* **123**, 1.
8. Stephen, A. B. T., Macleod, J. K., Palni, L. M. S. and Letham, D. S. (1985) *Phytochemistry* **24**, 2611.
9. Tay, S. A. B., Palni, L. M. S. and MacLeod, J. K. (1987) *J. Plant Growth Regul.* **5**, 133.
10. Pedersen, M. (1973) *Physiol Plant.* **28**, 101.
11. Featonby-Smith, B. C. and Van Staden J. (1984) *Bot. Mar.* **27**, 527.
12. Koshimizu, K. and Iwamura, H. (1986) in *Chemistry of Plant Hormones* (Takahashi, N., ed.), p. 153. CRC Press, Boca Raton, Florida.
13. Letham, D. S. and Palni, L. M. S. (1983) *Annu. Rev. Plant Physiol.* **34**, 163.
14. Wang, T. L., Beutelmann, P. and Cove, D. J. (1981) *Plant Physiol.* **68**, 735.
15. Beutelmann, P. and Bauer, L. (1977) *Planta* **133**, 215.
16. Biddington, N. L. and Thomas, T. H. (1973) *Planta* **111**, 183.