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CYTOKININS IN SHOOTS OF THE CHESTNUT TREE

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Key Word Index—*Castanea* spp.; Fagaceae; chestnut; cytokinins; *trans*-zeatin; *trans*-zeatin riboside; *trans*-zeatin-*O*-glucoside: N^6-(3-methyl-but-2-enyl)adenine; N^6-(3-methyl-but-2-enyl)adenosine.

Abstract—Five cytokinins, *trans*-zeatin, 9-β-D-ribosyl-*trans*-zeatin, *O*-β-D-glucosyl-*trans*-zeatin, N^6-(3-methyl-but-2-enyl)adenine and N^6-(3-methyl-but-2-enyl)adenosine were identified in shoots of the chestnut tree.

INTRODUCTION

Chestnut trees (*Castanea* spp.) form galls when parasitized by larvae of *Dryocosmus kuriphilus* Yasumatsu, a cecidogenetic wasp [1]. In the previous paper [2], it was reported that an unknown factor(s) contained in the larvae might be an external cause to induce gall formation since the extract of the larvae induced a swelling when administered to the bud which was just beginning to sprout. On the other hand, the healthy shoot tissue contained a high level of cytokinin activity and a low level of auxin activity, which was totally different from the hormonal content found in the gall tissue [2]. This fact indicates that the hormonal balance found in the host plant itself could be one of inherent causes in the promotion of gall formation. A further attempt was made to purify and characterize endogenous cytokinins associated with the high cytokinin activity in the shoot tissue, resulting in the identification of five cytokinins.

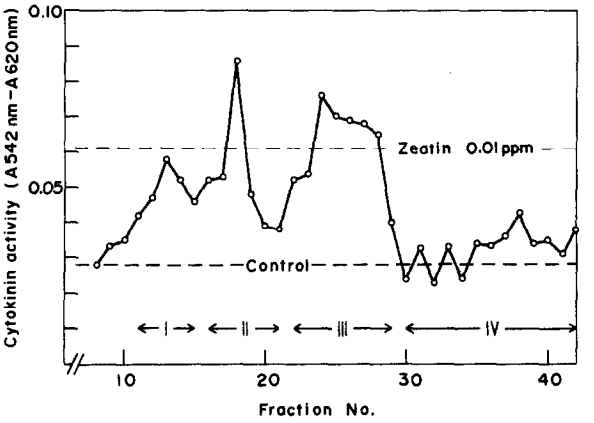
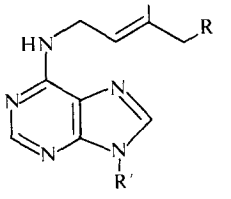


Fig. 1. Separation of cytokinin activity by PVP chromatography of the chestnut shoot extract. For details of procedure, see text. 18 g fr. wt equivalents were used for bioassay.

RESULTS AND DISCUSSION

An aqueous extract obtained by concentrating the ethanol extract of shoots and 1-year-old twigs was washed with ethyl acetate at pH 3 to remove impurities and fractionated on a Dowex 50W column. Active material was recovered by eluting with ammonia–ethanol. The total activity recovered from 1.7 kg of the plant material was estimated by the *Amaranthus* betacyanin test [3] to be *ca* 170 μg of zeatin equivalents. The active material was purified by insoluble PVP column chromatography [4], yielding four active fractions (I, II, III and IV) as depicted in Fig. 1.

Fraction I was purified by PC (solvents a and b) and silanized Si gel TLC (solvent c). Further purification by HPLC using LiChrosorb DIOL and then Nucleosil C18 afforded a cytokinin termed compound A, which showed the same R_t values as those of *O*-β-D-glucopyranosyl-*trans*-zeatin which had been isolated and identified from immature seed of *Dolichos lablab* [5]. A was hydrolysed with β-glucosidase, and the hydrolysate examined by PC and subsequent bioassay. The main activity was observed at R_fs indistinguishable from those of zeatin in both ammoniacal *sec*-butanol (solvent a) and borate buffer (solvent d). More rigorous evidence was obtained as follows. The enzymatic hydrolysis mixture was directly chromatographed by HPLC using Nucleosil C18, only one peak with the same R_t as that of *trans*-zeatin being observed. This peak was collected and analysed by GC–MS after trimethylsilylation. A peak with the same R_t (3.7 min) as that of $TMSi_2$ *trans*-zeatin showed a MS identical with $TMSi_2$ *trans*-zeatin: *m/e* 363 (M^+, 17%), 348 (23), 273 (89), 260 (100), 258 (27), 232 (38), 192 (32), 156 (45). From these results A was identified as *O*-β-D-glucopyranosyl-*trans*-zeatin (**1**).



	R	R
1	OGlc	H
2	OH	β-D-ribosyl
3	OH	H
4	H	β-D-ribosyl
5	H	H

Fraction II was purified by PC (solvent e) and the eluate from the active zone was analysed by GC–MS after trimethylsilylation. 9β-D-Ribosyl-*trans*-zeatin (**2**) was assigned to the responsible principle (compound B) based on the R_t (9.7 min) and MS of its $TMSi_4$ derivative: *m/e* 639 (M^+, 42%), 624 (33), 549 (71), 536 (73), 320 (41), 276 (19), 259 (53), 245 (57), 243 (41), 230 (67), 217 (91), 201 (100), 188 (72), 169 (18), 156 (78).

Fraction III was purified by Sephadex LH-20 chromatography [6] to afford two active fractions. The fast-moving major cytokinin was named compound C and the slowly-moving minor one compound D. They were separately purified by TLC. C was converted to its per-TMSi derivative, GC–MS of which showed, at the R_t of 3.4 min, a MS identical with that of $TMSi_3$ zeatin: *m/e* 435 (M^+, 20%), 420 (21), 346 (100), 332 (32), 304 (91), 292 (13), 272 (27), 264 (33), 192 (12), 191 (16), 156 (30), 131 (48), 129 (36), 127 (39). Thus C is identified as zeatin (**3**). Compound D was analysed by GC–selected ion monitoring (SIM). Since the chromatographic behaviour of this compound was indistinguishable from that of N^6-(3-methyl-but-2-enyl)adenosine (i^6Ado), the fragment ions at *m/e* 232 (base peak), 318 and 348 which are characteristic of $TMSi_3$ i^6Ado were selected for ion current monitoring. The relative intensities (36:6:7) of these peaks appearing at the R_t (4.9 min) of $TMSi_3$ i^6Ado were consistent with those of $TMSi_3$ i^6Ado, indicating that D is i^6Ado (**4**).

Fraction IV was purified by TLC. An active principle contained in the zone which had the same R_f as that of N^6-(3-methyl-but-2-enyl)adenine (i^6Ade) was termed compound E. GC–MS analysis after trimethylsilylation of this compound with R_t of 3.7 min showed a MS identical with that of TMSi i^6Ade: *m/e* 275 (M^+, 41%), 260 (67), 232 (100), 207 (39), 192 (35). This result was also substantiated by GC–SIM carried out by monitoring ions at *m/e* 275, 260 and 232. Thus the E was identified as i^6Ade (**5**).

It was reported that *trans*-zeatin and 9β-D-ribosyl-*trans*-zeatin were responsible for most of the cytokinin activity found in chestnut tree shoots, while *O*-β-D-glucosyl-zeatin, i^6Ade and i^6Ado were minor cytokinins. The latter two cytokinins, however, might have been present in higher quantity than described here, since these cytokinins should have been partially removed when extracted with ethyl acetate, which was used to remove impurity from the crude extract [7]. The level measured for glucosyl zeatin may not be far from the true content in the tissue despite the usage of Dowex-50 in the purification step, since cytokinin glucosides have been reported to be stable against this strong acid resin [8]. This study also demonstrates that less than a few μg of cytokinin can be definitely identified if properly selected purification methods are used in combination with GC–MS or GC–SIM.

EXPERIMENTAL

Bioassay. Betacyanin production by *Amaranthus caudatus* L. was used to measure cytokinin activity [3].

Chromatographic techniques. Toyo Paper Nos. 526 or 50 was used for PC. TLC plates were prepared with Merck silanized Si gel PF_{254} [9]. The following solvents were used for chromatography: (a) H_2O-satd *sec*-BuOH; (b) *sec*-BuOH–conc NH_4OH (4:1); (c) MeOH–H_2O (2:3); (d) 0.1 M borate buffer, pH 8.5; (e) *n*-BuOH–conc NH_4OH–H_2O (86:9:5). MeOH or 80% EtOH was used to elute the cytokinins.

GC–MS and GC–SIM were carried out according to the procedures already described [10] unless otherwise stated. TMSi derivatives were prepared by allowing samples to react with MeCN–BSA (2:1) at 70° for 10 min if not specified. 2% OV-1 packed in a glass column (3 mm × 1 m) was used if not specified.

HPLC. JASCO pumping system (SM10P20-10G15H-PG350D) connected to a detector unit, JASCO Uvi Dec-100 and an injection unit, Rheodyne model 7120 was used. Effluent was monitored at 269 nm. LiChrosorb DIOL (Merck, particle size 10 μm) and Nucleosil 7 C18 (Macherey-Nagel Co., particle size 7 μm) were packed with slurry solvents A and B (Nagel) respectively.

Plant material and extraction. Shoots and 1-year-old twigs were collected between April and May from chestnut trees grown in the Fruit Research Station (Hiratsuka). After removing leaves, stems (1.7 kg) were cut into small pieces, which were homogenized in 5 l. EtOH. Further extraction was made using 5 l. 75% EtOH × 2. Combined EtOH extracts were concd *in vacuo* to 0.8 l. aq. residue, which was washed with 0.6 l. of EtOAc × 3 to give the aq. soln. This, after removing EtOAc *in vacuo*, was run through a column (4 × 20 cm) of Dowex 50 × 8 (100–200 mesh, H^+ form). The column was washed with H_2O (0.8 l.), 95% EtOH (1 l.) and cold 6 N NH_4OH in 50% EtOH (1 l.). The last eluate only showed cytokinin activity in the *Amaranthus* test.

Purification of active compounds. The active fraction eluted from the ion exchange column was purified with a column (2.8 × 26 cm) of insoluble PVP (Tokyo Kasei) [4]. M/75 NaPi buffer, pH 6.4 was used for elution and collected in 15 ml fractions. Based on biological activity, four combined fractions were obtained: F-I (elution vol: 150–255 ml), F-II (225–315 ml), F-III (315–435 ml) and F-IV (435–630 ml).

Purification of F-I. F-I was purified on Toyo No. 526 paper using solvent a (active zone; R_f 0.32–0.48) and then solvent b (active zone: R_f 0.07–0.16). Further purification by TLC (solvent c) gave an active fraction (R_f 0.43–0.65). This fraction was purified by HPLC using LiChrosorb DIOL column (4 mm × 25 cm) (solvent, 10% MeOH in $CHCl_3$; flow rate 2 ml/min). A peak eluting at 4.2 min was collected. Final purification was carried out with Nucleosil 7 C18 column (4 mm × 25 cm) (solvent, 30% MeOH in H_2O; flow rate 2 ml/min) to afford compound A with the R_t of 4.3 min (2 μg based on the HPLC peak height).

Purification of F-II. F-II was chromatographed by PC using Toyo No. 50 paper and solvent e. The extract from the zone between R_fs 0.5 and 0.65 contained a cytokinin-active principle compound B (6 μg zeatin equivalents from bioassay). This extract was silylated with MeCN–BSA–TMCS (10:5:1), then analysed by GC–MS using 3% OV-1 (oven temp. 287°).

Purification of F-III. F-III was purified by Sephadex LH-20 column chromatography (column size, 2.7 × 28.5 cm; solvent, 35% EtOH) to give a main active fraction (elution vol. 201–237 ml) and a minor one (237–273 ml). The former was purified by TLC (solvent c) to give an active zone (R_f 0.32–0.42) which contained compound C (less than 10 μg zeatin from bioassay): cf. R_f of zeatin = 0.37, R_f of i^6Ado = 0.24). This was analysed by GC–MS using 3% OV-1 (oven temp. 250°). The latter fraction was purified by the same procedure, yielding a UV-absorbing active zone (R_f 0.21–0.27) which contained compound D (0.5 μg i^6Ado equivalent from UV absorption). This was analysed by GC–SIM (oven temp. 250°).

Purification of fraction IV. TLC was used to purify fraction IV, yielding a main active zone (R_f 0.11–0.21) which contained compound E (0.5 μg kinetin equivalent from bioassay). The eluate from this zone was subject to GC–MS (oven temp. 200°) and GC–SIM (oven temp. 190°).

Analysis of enzymatic hydrolysate of compound A. Experiment a. A was treated overnight at 37° with 0.1 mg β-glucosidase (Sigma) in 1 ml 0.05 M NaOAc buffer, pH 5. The reaction mixture was evapd to dryness and extracted with EtOH. The R_f of the activity

was observed at R_f 0.42–0.51 in solvent b (cf. R_f of zeatin = 0.47, R_f of zeatin riboside = 0.55) and at R_f 0.49–0.58 in solvent d (cf. R_f of zeatin = 0.54, R_f of zeatin riboside = 0.84). *Experiment b.* A was allowed to react with 40 μg of the enzyme in 40 μl of the buffer for 3 hr at 37°. This was directly purified by HPLC using Nucleosil 7 C18 column (solvent, 27% MeOH; flow rate, 2 ml/min). A peak at R_t 9.8 min was collected and subjected to GC–MS (oven temp. 205°).

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