A NEW CYTOKININ METABOLITE

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SUMMARY 8-[^{14}C]-zeatin is rapidly metabolised by cytokinin requiring soybean callus tissue to a number of compounds. The major metabolite has been tentatively identified as 6-(4-O-β-D-glucosyl-3-methyl-trans-2-butenylamino)purine.

INTRODUCTION

The metabolism of zeatin has been studied extensively in several plant species (1,2,3). However nothing is known of the metabolic fate of this highly active cytokinin in cytokinin requiring plant callus cultures. Fox et.al. (4,5) have studied the metabolism of the synthetic cytokinin 6-(benzylamino)purine in cytokinin requiring soybean callus tissue and have tentatively identified 6-(benzylamino)-7-glucofuranosylpurine as the major metabolite. Since this compound persists in the tissue for a relatively long period of time after all the applied 6-(benzylamino)purine has disappeared Fox (6) has suggested that it may represent the active form of the cytokinin.

The purpose of the present study was to ascertain whether or not the naturally occurring and more active cytokinin zeatin, showed a similar pattern of metabolism in this system. Zeatin is the most active cytokinin known in inducing cell division in cytokinin requiring callus cultures so it might be expected that if 7-glucoside formation is of any general functional significance as regards the mode of action of cytokinins, zeatin-7-

glucoside would be an important metabolite of zeatin in this system.

Parker and Letham (2,3) have identified a 7-glucoside of zeatin as the major metabolite of [^{3}H]zeatin in de-rooted radish seedlings and a 9-glucoside as a metabolite in corn seedlings. The investigation reported here has revealed that the metabolism of zeatin by cytokinin requiring soybean callus does not lead to the formation of either of the above compounds but that the major metabolite present after 24 h incubation is the side chain glucoside 6-(4-O-β-D-glucosyl-3-methyl-trans-2-butenylamino)purine.

MATERIALS AND METHODS

Cytokinin requiring soybean callus tissue was obtained and cultured using the methods of Miller (7). Pieces of callus (1 gm) were excised from stocks grown on kinetin (1 mg/l) and cultured for 5 days on a kinetin free medium. An aqueous solution (100 µl) containing 0.1 µg of 8-[^{14}C]-zeatin (1 mc/mmole) was applied to the surface of each of 500 pieces of callus. After 24 h the callus pieces were dropped into 80% methanol at 70°C, the solution cooled to 0°C and the tissue homogenised. The methanolic extract was filtered, reduced to dryness, dissolved in a small volume of water at pH 8.0 and passed through a DEAE-cellulose column (HCO_3^- form) which was washed with water. The water washings were reduced to a small volume, acidified to pH 3.0 and passed through a cellulose phosphate column (NH_4^+form). After washing with water the column was eluted with 1N ammonia. The ammonia eluate was evaporated to dryness, dissolved in a small quantity of 35% ethanol and chromatographed on a LH-20 Sephadex column eluted with the same solvent.

Thin-layer chromatography (TLC) was carried out on Merck prepared plates (GF_{254} silica gel, 20 x 20 cm x 0.5 mm).
Chromatographic solvents used were (v/v):
A) butan-1-ol/acetic acid/water (12:3:5)
B) butan-1-ol/14N ammonia/water (6:1:2) upper phase
C) butan-2-ol/14N ammonia (4:1)
D) chloroform/methanol (9:1)

RESULTS

The distribution of radioactivity in the ammonia eluate from the DEAE-cellulose column after chromatography on a LH-20 Sephadex column eluted with 35% ethanol is shown in Fig. 1.

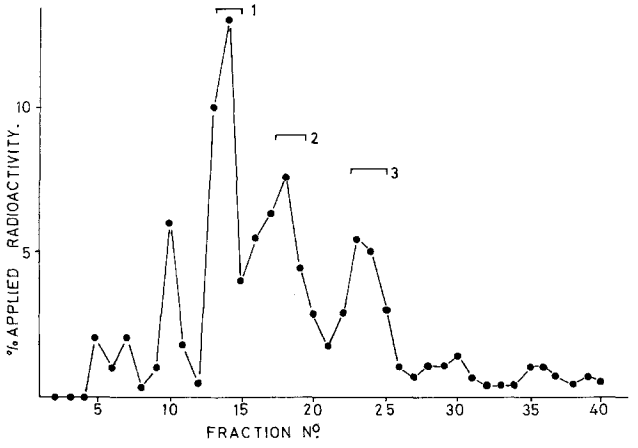

Fig. 1. Distribution of radioactivity in ammonia eluate of cellulose phosphate column after chromatography on LH-20 Sephadex eluted with 35% ethanol. Bars indicate the elution volumes of synthetic markers:

1. Zeatin-7 and -9-β-D-glucopyranoside
2. Zeatin riboside
3. Zeatin

The major peak of radioactivity was subjected to two preparative TLC separations in solvents A and B. Autoradiography revealed a single major zone of radioactivity in each case (R_f 0.40 solvent A and R_f 0.20 solvent B). The material was finally purified by chromatography on exhausitvely washed paper (Whatmann 3MM) in solvent system C (chromatogram developed 3X). A total quantity of 12 μg of material (estimated from radioactivity) was isolated.

In the solvent systems used the major zone of radioactivity co-chromatographed with synthetic zeatin-7-β-D-glucopyranoside. However the UV spectral characteristics of the metabolite clearly showed that it was not a 7- or 9-substituted 6-aminopurine.

The marked similarities between the UV spectra of the

metabolite and those of zeatin suggested that it was the result of a modification of the side chain of the zeatin molecule. Attempts to GLC the compound as a TMS derivative resulted in 25% of the radioactivity injected onto the column appearing as a single peak which was identified as TMS zeatin by GCMS. The remainder of the radioactivity bled off the column over a period of time suggesting that the TMS derivative was incompletely formed or was thermally unstable. Treatment of the compound with highly purified β-glucosidase followed by TLC in solvent system B revealed the presence of a single radioactive spot which co-chromatographed with authentic zeatin. No starting material could be detected after β-glucosidase treatment. However, treatment of the metabolite with α-glucosidase gave only starting material. The low resolution mass spectrum of the product of β-glucosidase treatment was identical with that of authentic zeatin showing principal peaks at 219 (M+), 202, 201, 188, 186, 160, 148, 136, 135, 120, 119, 108 and 93.

TABLE 1

Spectral characteristics of soybean metabolite compared with zeatin, zeatin-7-glucoside (Z-7-G), and zeatin-9-glucoside (Z-9-G).

	λ_{max} (nm)		
Compound	pH 7.0	pH 11.0	pH 1.0
Metabolite	270	276, 284(s)	274
Zeatin	269	275, 284(s)	274
Z-7-G	277	277	283
Z-9-G	268	268	268

The results presented suggested the metabolite was the side chain O-β-D-glucoside of zeatin. The trans configuration of the side chain was established co-chromatography of the product of enzymatic hydrolysis with authentic trans-zeatin in solvent system D (8).

From the above date it was tentatively concluded that the major metabolite of zeatin in cytokinin requiring soybean callus tissue after 24 h incubation is 6-(4-O-β-D-glucosyl-3-methyl-trans-2-butenylamino)purine. The synthesis of this compound is at present under investigation.

DISCUSSION

It has been suggested that the formation of 7-glucosides is of functional importance with regard to the mode of action of some cytokinins in plant callus systems. It has further been suggested that the mode of action of the anti-cytokinin 4-furfurylamino-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine may involve competitive inhibition of the enzyme system involved in 7-glucosylation (9). The observations reported here would suggest that since zeatin (the most active cytokinin known) is not metabolised to a 7-glucoside by cytokinin requiring soybean callus tissue, it is unlikely that the formation of 6-(benzylamino)-7-glucofuranoside observed by Fox in this system is related in any way to the mode of action of the cytokinin. It may well be that the formation of cytokinin glucosides is more representative of attempts by plant tissue to inactivate these very physiologically active compounds, although in certain circumstances they may function as storage or transport forms of the cytokinin as has been suggested by Parker and Letham (2,3). In general however, it is felt that the formation of cytokinin 7-glucosides by cytokinin requiring soybean callus

cultures is of little functional significance. Although the side chain glucoside reported here persists in the tissue for a considerable length of time after the applied zeatin has been metabolised, it is felt that its production is more likely to involve an inactivation response by the tissue than the production of an active form of the cytokinin.

It is of interest to note that while the side chain glucoside is easily cleaved by β-glucosidase both zeatin-7-β-D-glucopyranoside and zeatin 9-β-D-glucopyranoside are resistant to this enzyme, presumably because of their C-N glycosidic linkages. This would suggest that the side chain glucoside is more likely to be the compound reported by Yoshida et.al., than zeatin-9-glucoside as they suggest (10).

Initial observations indicate that 6-(4-O-β-D-glucosyl-3-methyl-trans-2-butenylamino)purine is the major endogenous cytokinin in leaves of Phaseolus vulgaris (11).

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