Isolation and Identification of trans-Zeatin from the Roots of
Raphanus sativus L. cv. Sakurajima

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Cell division-inducing substances in the roots of Raphanus sativus L. cv. Sakurajima were extracted with methanol and fractionated. Tobacco callus bioassay showed most of the cytokinin activity to remain in a basic fraction which is soluble to both n-butanol and hot ethyl acetate. On further successive separation, i.e., Celite column chromatography, Sephadex LH-20 column chromatography and silica gel tlc, this fraction afforded one of the components as fine needles to which most of the activity may be attributed. This was identified as trans-zeatin based on thin-layer chromatographic behavior and UV and MS spectral data.

Zeatin, a cell division-inducing substance, was first isolated by Letham1) in 1963 from young kernels of Zea mays. The report was followed by isolation of other cytokinins including ribosylzeatin from immature sweet corn,2) glucosylzeatin from coconut milk3) and roots of a rice plant,4) (-)-dihydrozeatin from immature yellow lupin seeds5) and 6-(o-hydroxybenzylamino)-9-β-D-ribofuranosylpurine from poplar leaves.6) The occurrence of cytokinin-like substances has also been suggested chromatographically in germinating peas,7) apple fruitlets,8) tomato fruits9) and carrot roots.10) These facts indicate that cytokinins are closely related to development of tissues both in young and growing stages. While naturally occurring cytokinins have ever been isolated mostly from immature tissues, recent progress of isolation technique has given a light on the identification of physiologically significant cytokinins even if they are in growing tissues such as those of Raphanus root where the content of cytokinins is expected to be so slight.

In Raphanus sativus, tuber growth is associated with massive development of parenchyma in the inner tissues of the tap root and hypocotyl. Among Japanese cultivars the root thickening of Raphanus sativus L. cv. Sakurajima is the greatest and the root forms a spherical ball as large as 30 ~ 40 cm in diameter. Hitherto endogenous plant hormones controlling root thickening of this species have not been examined. Assuming cytokinin as one of the hormones, we started the isolation and identification of principles having cytokinin activity from thickening roots harvested four months after sowing. The detail of the work will appear in this paper.

An aqueous methanolic extract from 670 kg of detached roots of R. sativus L. cv. Sakurajima was adjusted to pH 3 and washed with ethyl acetate to remove fatty materials. The aqueous solution was re-adjusted to pH 3 and passed through a column of Dowex 50W (H+ form). After washing with water, basic components adsorbed on the column were eluted with 2 N ammonia. Bioassay at this stage showed that the basic eluate has most of the activity. The eluate was concentrated below 45°C and partitioned with n-butanol. Most of the activity was transferred to n-butanol layer.

* With respect to solvent system see EXPERIMENTAL.
FIG. 1. Cytokinin Activities of Fractions Obtained from Basic Eluate(a), n-Butanol Layer(b) and Aqueous Layer(c) by Paper Chromatography.

An aliquot of the basic eluate, n-butanol layer and aqueous layer was streaked on about ten sheets of Whatman No. 1 paper (3 cm x 40 cm) and developed with solvent A in an ascending manner. Each sheet was divided into ten Rf strips, extracted with hot methanol-water (1:1) and the extract was screened by bioassay.

showed remarkable activity at Rf 0.7~1.0 and significant activity at Rf 0.4~0.7 (Fig. 1). In contrast, the aqueous layer showed weak activity at Rf 0.4~0.6, 0.7~0.8 and 0.9~1.0. After removal of the n-butanol in vacuo, the residue was extracted with boiling ethyl acetate and the cell division-inducing factors were transferred to the ethyl acetate-soluble fraction (4.7 g).

The fraction was submitted to Celite column chromatography according to the method of Robins et al. The column was eluted with water-saturated ethyl acetate at 20°C and monitored by UV absorbance at 270 nm. Fraction A, B and C in Fig. 2 exhibited cell division activity. The elution volumes of fraction B and C coincided with those of authentic zeatin and ribosylzeatin respectively. The fastest-eluting active component in fraction A was therefore neither zeatin nor ribosylzeatin. The cytokinin activity of the three active fractions was in the order B>A>C. The most active fraction, B, was further purified by Sephadex LH-20 column chromatography (Fig. 3). Strong activity was found only in the tenth fraction. The elution volume of the fraction coincided with that of authentic zeatin. The active fraction was streaked on a silica gel plate which was developed with solvent B. Extract from

FIG. 2. Celite Column Chromatography of Ethyl Acetate-soluble Fraction.

Ethyl acetate-soluble fraction: 51 mg; column: 3.3 cm x 50 cm; Celite 545: 200 g; solvent: water-saturated ethyl acetate (20°C); flow rate: 55 ml/hr; volume per tube: 17 ml; A: adenine; Z: zeatin; Z-R: ribosylzeatin. Portions of 1/10 (□) and 1/100 (▲) of each fraction were subjected to tobacco callus test.
the zone at Rf 0.61 - 0.69 showed the strongest activity and it was further submitted to silica gel tlc (solvent C). Six UV-absorbing spots were detected and remarkable cytokinin activity was located at the zone of Rf 0.35 - 0.42 which coincided with the Rf value of zeatin. For the final purification, the active component at Rf 0.35 - 0.42 was further chromatographed on a preparative silica gel plate with solvent A. The active zone gave 0.2 mg of fine needles from an aqueous solution.

The active component proved to be different from cis-zeatin on silica gel tlc developed with solvent F[11] and also different from (±)-dihydrozeatin on cellulose tlc developed with solvent G. On the contrary, the Rf values of the active component were identical with trans-zeatin in five solvent systems as shown in Table I.

UV spectra of the active component in neutral, acidic and alkaline ethanolic solutions were identical with those of trans-zeatin as shown in Table II. It showed the following mass spectrum: m/e 219 (M⁺), 202, 188, 160, 149, 136, 135, 119 and 92 which is characteristic of trans-zeatin and cis-zeatin, the two geometrical isomers being indistinguishable on MS spectrum alone (Fig. 4).

It has been shown in many different plants that cytokinins are produced in root and exist there. The present paper has shown the presence of trans-zeatin as a major cytokinin in the Raphanus root. It will be interesting therefore to study the physiological effects of trans-zeatin and of related substances on root thick-
FIG. 4. Mass Spectra of the Active Component and Authentic trans-Zeatin. Direct inlet method, 70 eV.

ten of this species. Further efforts to identify the remaining active components in fraction A and C are under progress.

EXPERIMENTAL

Chemicals and instruments

Except for methanol, all solvents of analytical grade were distilled before use. Zeatin and ribosylzeatin each of which is a mixture of cis and trans isomers were purchased from Sigma Chemical Co. trans-Zeatin, cis-zeatin and (±)-dihydrozeatin were synthesized according to methods reported previously. UV spectra were recorded on a Hitachi double beam spectrophotometer, model 200-10 and mass spectra were obtained using a Hitachi mass spectrometer, model RMU-4.

i) Bioassay. Cytokinin activity of each fraction was tested with tobacco callus cultured on Linsmaier and Skoog's medium. The fresh callus grown for about 40 days at 29°C was weighed. There were four replicates for each assay.

ii) Paper chromatography and thin-layer chromatography. The following solvent systems were used.

A: n-butanol–water–28% ammonia (172: 18: 10)
B: n-butanol–acetic acid–water (12: 3: 5)
C: 2-butanol saturated with water
D: isopropanol–water–25% ammonia (7: 1: 1)
E: ethyl acetate–n-butanol–water (4: 1: 1); upper phase
F: chloroform–methanol (9: 1)
G: water

iii) Isolation of trans-zeatin from the roots of R. sativus L. cv. Sakurajima. A total of 670 kg of the fresh roots grown for four months from August to December in 1975 were harvested and washed with water. Each 10 kg of the roots was chopped into pieces and immediately ground into a vessel containing 10 liters of methanol. After standing for more than a week, a clear methanolic extract was separated from solid materials by filtration using press filter and the extract was centrifuged. The solid materials were extracted with 2 liters of fresh methanol in a similar way. The methanolic solutions thus obtained were combined, concentrated at reduced pressure to ca. one sixth of the total volume and stored at 5°C.

A portion of 8 liters of the concentrated extract which proved to contain ca. 2 kg of dry matter was adjusted to pH 3 with 6 N hydrochloric acid with vigorous stirring and washed twice with ethyl acetate (200 ml). The aqueous solution was re-adjusted to pH 3 and passed through a column of Dowex 50W×8 (H⁺ form, 50~100 mesh, 4 liters, 11 cm x 100 cm) and washed with 40~50 liters of deionized water until the washings were no longer acidic. The effluent and washings were combined. Basic components were eluted with 10 liters of 2 N ammonia and the eluate was concentrated in vacuo below 45°C to ca. 800 ml to give an almost neutral aqueous solution. The solution was extracted five times with 250 ml of n-butanol and the extracts centrifuged at 3000 rpm for 10 min for phase separation. The n-butanol extracts were com-
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1. Combined and evaporated in vacuo to give 2.9 g of pale yellow powder. The powder was extracted with 120 ml of boiling ethyl acetate for 10 min. Insoluble precipitates were filtered out while hot, ground and extracted again. The combined filtrates were dried to give 0.4 g of the ethyl acetate-soluble fraction. The fractionation procedure was repeated with additional lots to yield a total of 4.7 g of the ethyl acetate-soluble fraction from 670 kg of tubers.

2. For preparative purpose, an amount of 0.5–2 g of the fraction was dissolved in ethyl acetate-saturated water on heating. The solution was mixed with 5 g of Celite 545 and added to the top of a column (200 g of washed Celite 545 mixed with 93 ml of ethyl acetate-saturated water) and eluted with water-saturated ethyl acetate at 20°C at a flow rate of 50 ml/hr. Elution was monitored by the absorbance at 270 nm. The eluted fractions were combined into nine groups and one tenth or one hundredth portion of each combined fraction was submitted to bioassay. A quarter of combined fraction B was mixed with 4 ml of hot methanol-water (1:1) and filtered. The filtrate was charged on a column (2.3 cm x 80 cm) packed with Sephadex LH-20 and eluted with water-saturated ethyl acetate at a flow rate of 30–40 ml/hr. Each 11 ml of eluate was collected. After elution, fractions were combined into seven groups as shown in Fig. 3 and submitted to bioassay. The active tenth fraction was accumulated by repetition of the fractionation and streaked on a Merck 60F254 silica gel plate and developed with solvent A. After drying in air, the plate was divided into five fractions and each fraction was extracted twice with 4–5 ml of methanol-water-28% ammonia (75:25:8) at room temperature for several hours. The combined methanolic extracts an aliquot of which was subjected to bioassay were concentrated to dryness in vacuo. The most active fraction at Rf 0.61–0.69 was dissolved in a small amount of water to give 0.2 mg of crystalline zeatin.

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