Studies on Growth of Japanese Pear Fruits
I. Isolation of Zeatin and its Related Compound from Immature Japanese Pear Fruits

Masanori OHKAWA
Faculty of Agriculture, University of Nagoya, Chikusa, Nagoya

Summary
Immature fruits of Japanese pear, Pyrus serotina, were picked and homogenized with 70% ethanol. The homogenate was extracted with 70% ethanol three times and filtrated. The filtrate was concentrated, the residue adjusted to pH 8.4 with 1 N NaOH and then extracted with n-butanol. The butanol phase was evaporated and aqueous extract of the residue was added to a Dowex-50(H+ form) column and eluted with water, 1 N NH₄OH and 5 N NH₄OH successively. Cytokinin activities in the three fractions were tested by tobacco(Wisconsin No. 38) pith callus assay. Cytokinin activities were detected in both 1 N and 5 N NH₄OH fractions, but not in the water. Based on these results, the NH₄OH fractions (Am-fraction) were combined and analyzed by microcrystalline cellulose thin layer and gas liquid chromatographies. The fraction separated by TLC for the bioassay of cytokinin revealed activities at Rf values 0.5 and 0.7. Analyses of TMS derivative of Am-fraction by GLC gave four peaks. Mixture of the TMS derivative with that of authentic zeatin analyzed on GLC revealed that peak 2 coincides with zeatin, and peak 1 was considered to be dihydrozeatin.

Introduction
Several natural cytokinins have been extracted from immature seeds of sweet corn by MILLER(12) and LETHAM(4, 5, 6), one of which is zeatin, having a chemical structure 6-(4-hydroxy-3-methyl-2-trans-butenylamino) purine(4). Since then, zeatin and its related compounds have been isolated from various higher plants and microorganisms(15).

Cytokinins are known to have many physiological effects on developing plant organs. LETHAM(9) isolated two cytokinins from plum fruitlet and carrot phloem explants which promoted cell division in the absence of exogenous auxins. When tobacco pith cells were treated with the kinetins in a low concentration, cell enlargement was promoted(8). The levels of these endogenous cytokinins were found to be higher in immature fruits and root tips than in other organs of the plant(8).

This paper deals with the cytokinins isolated from immature fruits of Japanese pear, Pyrus serotina, are reported.

Materials and Methods
Immature fruits developed about 1 cm in diameter were collected from bearing trees of Japanese pear, Pyrus serotina, and homogenized with a small quantity of 70% ethanol. The homogenate was steeped in 2000 ml of 70% ethanol at room temperature for 24 hrs after which the mixture was filtered through a Buchner funnel. The residue was extracted twice with additional fresh solvent. The filtrates were combined and treated as follows (also see Fig.1):

Fractionation
The filtrate was concentrated from 6000 ml to 500 ml in vacuo at 50°C with a rotary evaporator. The residue was adjusted to pH 8.4 with 1 N NaOH and extracted three times with 300 ml of n-butanol. The combined butanol phase was evaporated to dryness in vacuo at 50°C with a rotary evaporator and then taken up into 50 ml of water. fifteen ml of aqueous extract were added to a Dowex-50 (H⁺ form) column (3.5 cm×40 cm) and eluted with 500 ml of water, 1 N NH₄OH and 5 N NH₄OH.
NH₄OH successively. The volumes of water, 1 N NH₄OH and 5 N NH₄OH eluates were reduced to 15 ml in vacuo.

Bioassay for cytokinin activity.

The cytokinin activities in the three fractions were tested by tobacco (Wisconsin No. 38) pith callus assay. After twenty ml of the basal medium of RM-1964(10) were placed in 50 ml volume Erlenmeyer flasks; and the content was autoclaved and cooled, one ml of each fraction was added to individual flasks using a Millipore filter apparatus. Three callus explants were transferred aseptically to each flask, then cultured in the dark at 27°C. Explants were weighed 21 days after transfer and their growth rate calculated as follows: Δ weight/initial weight.

Paper chromatography.

One ml of Am-fraction was spotted on Toyo No. 51-A filter paper and the chromatogram developed by ascending chromatography using n-ButOH : NH₄OH (4 : 1, v/v) as solvent. After the solvent front moved about 45 cm from the origin, chromatogram was dried and cut into ten strips of equal width. The pieces were extracted twice with 10 ml of water. The volume of the extract was reduced to 5 ml in a freeze-drier of which one ml was used for bioassay.

Thin layer chromatography.

A half-ml aliquots of the Am-fractions were separated with microcrystalline TLC using water as solvent. On drying, the chromatogram was divided into ten zones which were scraped separately into test tubes. The contents were extracted twice with 10 ml of water, and then the volume reduced to 5 ml with a freeze-drier of which one ml was assayed.

Gas liquid chromatography.

Aliquots of the Am-fraction were added to a mixture of bis (trimethyl silyl) acetoamide and acetonitril (1 : 3, v/v) and heated at 60°C for 15 min. Two or three μl of silylized Am-fraction and authentic compounds were injected into a Nihon Denshi Model 1100 gas chromatography apparatus equipped with a stainless steel column (3 φ X 2 m) packed with 3% SE52 on 80-100 mesh Diatoport S support. The regulated temperatures were: column, 210°C or 250°C isotherm; injector, 230°C, and flame ionization detector, 250°C. Retention time of the unknown is expressed as relative values to the pyrene and kinetin(13).

Results

Cytokinin activities on paper chromatograms were detected in both 1 N Am- and 5 N Am-fractions at Rf 0.6, and low at Rf 1.0 (Fig. 2), but not in the water (Fig. 3). The material at Rf 0.6 zone coincided with authentic zeatin. Based on these results, the Am-fraction was combined and analyzed. Am-fraction separated

---

Fig. 1. Flow chart of fraction of cytokinin-active substances extracted from immature Japanese pear fruits.

Fig. 2. Histogram of cytokinin-activity obtained by paper chromatography of 70% ethanol extracts from immature Japanese pear fruits. Chromatogram developed with n-butanol : NH₄OH (4 : 1, v/v). Letter Z at the top of the middle histogram indicates the location of zeatin.
by microcrystalline cellulose thin layer chromatography and assayed for cytokinin revealed activities at Rfs 0.5 and 0.7 (Fig. 4). Analyses of TMS derivative of Am-fraction by gas liquid chromatography gave four peaks; peak 1, 2 and 3 being detected at 210°C and peak 4 at 250°C (Table 1). Mixture of the TMS derivatives of Am-fraction and authentic zeatin analyzed on GLC revealed that peak 2 coincides with zeatin. The amount of material in peak 1 is greater than that of peak 2.

**Discussion**

Eversince zeatin, an active growth substance, was isolated from immature sweet corn kernels, investigators have been successful in finding endogenous cytokinins in various species of higher plants(8). They are thought to be synthesized within roots(14), immature fruits and shoot apices of plant(8).

Short(14) reported that the seedling roots of pea contained zeatin and its related compounds. Cytokinin content was found to be high in the extreme 1 mm segment of excised tips, decreasing in concentration in the 1–5 mm zones; no activity was detected in segments of the 5–20 and 20–40mm distances from the tips. The 1 mm zone is quiescent; no cell division occurring. Short(14) attributed this behavior to a supra-optimal concentration of cytokinins and suggested that the root tip was a center in synthesis of these hormones.

However, endogenous cytokinins have been separated from young persimmon fruits(11), avocado(1, 2), plum fruitlets(7), and apple(9). Blumenfeld found that seeds, especially the endosperm, of developing avocado fruit contained very high levels of cytokinin(1). Cytokinin activities were also found in the mesocarp of avocado fruit, the level of which correlated positively with the cell division and the fruit growth rates(2).

Extract of developing apple seeds was much more active than that of the surrounding tissues, and thus seeds appeared to be an important site of cytokinin biosynthesis (9).

The present investigation reveals that immature fruits of Japanese pear contain high levels of cytokinin. The origin of these growth promoting substances, however, remains to be determined. Cytoki-
nins are known to promote the transport, accumulation and retention of metabolites in developing plant tissues and organs(8). Therefore, endogenous cytokinins play an important role in the development of pear fruits.

The Japanese pear fruit contains two cytokinins; one being identical with zeatin in respect of mobility on paper and TL chromatographies and retention time with GLC; and another is considered to be dihydrozeatin according to data of other workers(3,13).

Acknowledgment

I wish to thank Dr. H. Torikata, professor of Horticulture, University of Nagoya, and Dr. K. Ryugo, Professor of Pomology, University of California, Davis, for their valuable advices and revising English. I am also grateful to Dr. S. Matsubara, Kyoto Pref. University, for supplying tobacco pith callus to me and Mr. S. Torii for his technical assistance.

Literature Cited

日本ナシの果実の発育に関する研究（第１報）
日本ナシの未熟果に含まれるゼアチンならびにその関連化合物の分離

大川 勝德
（名古屋大学農学部）

摘 要

日本ナシの未熟果に含まれるサイトカイニンについて研究した。
未熟果からのエタノール抽出部をペーパークロマトグラフィーで分画し、タバコ（Wisconsin No.38）細胞カルスでサイトカイニンの活性を検定した。その結果 Rf 0.6 のバンドに強い活性を認めた。

エタノール抽出部をさらに pH 8.4 に調節し、n-プロタノールで抽出した。ついて n-プロタノール抽出部を Dowex-50 (H⁺) のイオン交換樹脂に吸着させ、水およびアンモニアの順で溶出し、それらのフラクションのサイトカイニンの活性を検定した。その結果アンモニアフラクションに強い活性を認めた。

活性のあるアンモニアフラクションをさらに試験管セールロースの薄層クロマトグラフィーおよび SE₃₄ をカラムとしたガスクロマトグラフィーで分析した結果、サイトカイニンとしてゼアチンの存在を認めた。またゼアチン関連化合物であるジヒドロゼアチンの存在も推定された。