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Water-Soluble Cytokinins in Blanching Water of Bamboo Shoots

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Abstract

The blanching water of bamboo shoots contained several kinds of cytokinins which stimulated the growth of tobacco callus *in vitro*. Water-soluble cytokinin among them was purified partially by *n*-butanol partition, ion-exchange Dowex 1 column and Sephadex LH-20 column and examined for its properties by using paper chromatography, barium precipitation and hydrolysis with alkaline phosphatase. The water-soluble cytokinin exhibited chromatographic behaviours different from those of zeatin and its riboside. Results obtained in the present study support a tentative conclusion that the water-soluble cytokinin in the blanching water of bamboo shoots is likely to zeatin ribotide. The aqueous fraction equivalent to 1 liter of the original blanching water contained a cytokinin comparable to 2×10^{-5} mole of zeatin ribotide.

Introduction

Shortly after kinetin was isolated as a cell division factor, namely as a cytokinin, in plant tissue culture from a hydrolysate of herring sperm DNA,[1] it was reported that kinetin riboside, N^6-furfurylamno-9-β-D-ribofuranosylpurine, affected the cell division and viability of mammalian cells *in vitro*.[2] Thereafter, numerous cytokinin-active purine derivatives were shown to have antitumor activities in mammalian cell cultures.[3-11] For plant cells, cytokinins also have some regulative actions in metabolisms of nucleic acids and proteins.[12,13]

On the other hand, cytokinins have been shown to be present in a number of edible plants such as sweet corn kernels,[14-18] pea,[19] fruitlets, of apple,[20-24] quince, peach, plum, pear, pumpkin, marrow[20] and persimmon[25] and turnip root.[26] A cytokinin isolated from sweet corn kernels has been first identified as 6-(4-hydroxy-3-but-*trans*-enylamino) puince[27] and named zeatin.[14] Zeatin riboside, 6-(4-hydroxy-3-but-*trans*-enylamino)-9-β-D-ribofuranosylpurine, and its ribotide also have been isolated.[15,16] In addition, cytokinins have been found in tRNA from a large number of organisms.[28-33] In a few cases, cytokinin-active minor bases have been identified and located next to the anticodon of specific tRNA species.[28,30,32] These investigations have suggested that cytokinins, especially in riboside or water-soluble ribotide form, present in edible plants have some significant roles in metabolisms of nucleic acids and proteins in the cells of human being.

During the course of the studies on natural cytokinins, a preliminary experiment revealed that an extract of bamboo shoots exhibited a high cytokinin activity in the tobacco callus bioassay. Therefore, partial purification and characterization of cytokinins, with special reference to water-soluble cytokinins, in the blanching water of bamboo shoots was attempted in the present study.

Materials and Methods

Blanching water of bamboo shoots — The blanching water of bamboo shoots used in this study was obtained from Kakimoto Cannary Co. LTD., Kizu, Kyoto. This blanching water was prepared by boiling 700 kg of bamboo shoots in 500 l of water for 2 hours and stored usually in a freezer as original blanching water.

Crude ethanol extract — For preparation of crude ethanol extract, 13 l of blanching water as one batch was concentrated to 65 ml by a rotary evaporator at lower temperature than 50℃ *in vacuo* (in the following experiments, evaporation and concentration of solutions were carried out in a rotary evaporator under these conditions) and added with 4 volumes of 95% ethanol. The precipitate was discarded, and the ethanolic supernatant was evaporated and added with water until 130 ml. The aqueous solution thus obtained was fractionated by adding with equal volume of petroleum ether. The petoleum ether layer was discarded. The aqueous layer was evaporated and dissolved in 130 ml of water. This solution which was at a concentration equivalent to 13 l of original blanching water per 130 ml was designated as "crude ethanol extract", was tested at several levels in cytokinin bioassay using tobacco callus and stored in a freezer for further purification. In this and following experiments, concentrations of the ethanol extract and purified fractions were expressed in terms of liter equivalents to the original blanching water per liter of medium (LE/l).

Partition with water-saturated n-butanol — Partition with water-saturated *n*-butanol was carried out by the method generally employed in purification of cytokinins. The crude ethanol extract (13 LE/130 ml, i.e. 13 l of original blanching water equivalents per 130 ml) was adjusted to pH 7.0 with NaOH solution, added with equal volume of water-saturated *n*-butanol and fractionated. This fractionation was repeated three times. The aqueous layer and the combined *n*-butanol fraction were evaporated and dissolved each in 65 ml of water (13 LE/65 ml, i.e. 13 l of original blanching water equivalents/65 ml). These two fractions were tested for their cytokinin activity at several levels and stored in a freezer for further purification.

Fractionation by ion exchange column— The aqueous fraction (12 LE/60 ml) was diluted with 40 ml of water and the solution was adjusted to pH 7.0. This solution was passed through a 2×22 cm column with ion exchange resin Dowex 1×2 (formate form, 50−100 mesh). The column was washed with 100 ml of water and the washing was combined with the effluent. Substances held by Dowex 1 was eluted with 200 ml of 4 M HCOOH. The effluent and the eluate were evaporated and dissolved each in 60 ml of water (12 LE/60 ml). These two fractions were designated as "effluent fraction" and "eluate fraction" respectively. Both fractions were tested for their cytokinin activity and used for further experiments.

Fractionation by Sephadex LH 20 column — Twenty ml of the eluate fraction (4 LE) was evaporated and dissolved in 2 ml of 35% ethanol. This solution was loaded on a 2×22 cm Sephadex LH-20 column and developed with 35% ethanol at a flow rate of 30 ml/hour. The

effluent volumes of 5 ml were collected with a fraction collector. Each 0.5 ml sample out of each 5 ml fraction was dried and dissolved in 50 ml of water and added to culture medium (2 times strength) for bioassay of their cytokinin activity.

Paper chromatography — The eluate fraction (0.1 LE/0.5 ml) was lined at 4 cm from the bottom of 40×40 cm Toyo filter paper No. 51 and developed ascendingly over 20 cm with three solvent systems, (A) water-saturated *n*-butanol, (B) water-saturated *sec*-butanol and (C) 0.03 M boric acid. Chromatograms were cut crosswise into ten equal sections according to Rf value. Each section was cut into pieces and eluted with 25 ml of water twice in a steam bath. Such 50 ml eluates were added to 50 ml of culture media (2 times strength) and tested for their cytokinin activity.

Barium precipitation — Precipitation by barium acetate was carried out by the method reported by Miller.[18] The eluate fraction (2 LE/10 ml) was diluted with 50 ml of water and the solution was adjusted to pH 9.0. Barium acetate solution (2 g/10 ml) was added, and precipitate was removed by centrifugation. Four volumes of 95% ethanol was added to the supernatant and kept overnight in a refrigerator. Precipitate produced during cooling was separated from the supernatant by centrifugation. The supernatant was concentrated to 40 ml and tested for its cytokinin activity. On the other hand, the precipitate was dissolved in 80 ml of 0.1 N H_2SO_4 by stirring at 50℃, and the solution was centrifuged. The residual precipitate was discarded. The supernatant was adjusted to pH 7.0, evaporated and dissolved in 40 ml of water (2 LE/40 ml). Such barium preciptate fraction was also tested at several levels for the cytokinin activity.

Enzymatic hydrolysis — Enzymatic hydrolysis was carried out as reported previously.[18] The eluate fraction (0.2 LE/1 ml) was developed with water-saturated *n*-butanol in a paper chromatography. The eluate from Rf 0−0.3 of the chromatogram was treated with chicken intestine alkaline phosphatase (Worthington Biochemicals) for 2 hours at 32℃. The reaction mixture included 0.01 M $MgCl_2$, 0.1 M Tris(hydroxymethyl)aminomethane at pH 8.2 and the enzyme at a concentration of 1 mg/ml. The hydrolysate which was prepared in 2.5 ml was developed with water-saturated *n*-butanol in a paper chromatography and the chromatogram was divided into 10 equal sections. The eluates obtained from these sections were tested for their cytokinin activity as described above.

Bioassay procedures — For tobacco callus (*Nicotiana tabacum* L. cv. Wisconsin No. 38) bioassay, the method reported previously [34,35] was used. The basal culture medium was the Linsmaier and Skoog medium [34] containing the mineral salts, 30 g/1 sucrore, 10g/1 agar, 100 mg/1 *myo*-inositol, 2 mg/1 IAA and 0.4 mg/1 thiamine-HCl. Aqueous solutions of all the samples to be tested were added to the basal media. After the agar was melted, the test media were distributed in aliquots of 20 ml in 50 ml conical flasks and autoclaved at 1.0 kg/cm^2 for 15 minutes. Three pieces of the tobacco callus (about 10 mg each in fresh weight) were implanted on the agar surface in each flask. Each experimental treatment contained 4 replicates of flasks. The flasks were maintained at 28℃ in the dark for 30 days, and then the fresh weight of tissues was determined.

Results and Discussion

Cytokinin activity of zeatin and kinetin was examined by the tobacco callus bioassay. As shown in Fig. 1, zeatin showed a detectable growth promotion at 10^{-10} M. The callus yield

increased with increasing concentration with the maximum yield at 4×10^{-8} M. Higher concentrations reduced the growth promotion. Kinetin stimulated slightly the growth at 4×10^{-9} M, and the maxmum callus yield was obtained at 10^{-7} M. Zeatin riboside, although not tested for its cytokinin activity in the present study, was about 100 times less active than zeatin and, zeatin ribotide is additionally, possibly more 10 times, less active than zeatin riboside.[11]

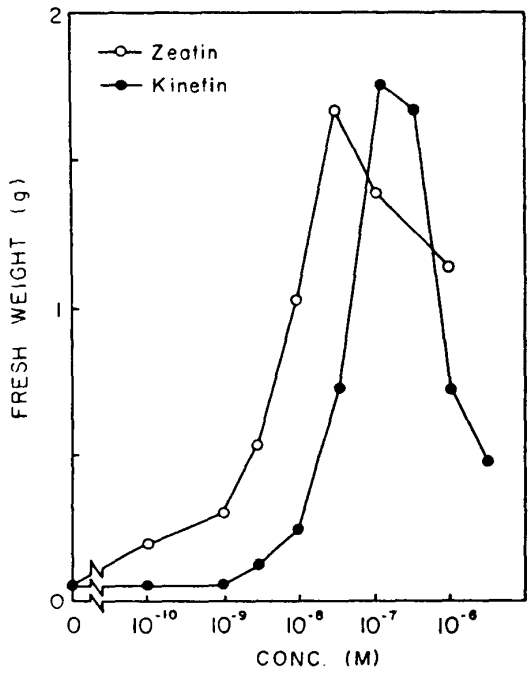

Fig. 1. Effects of zeatin and kinetin on the gorwth of tobacco callus *in vitro*.

By partition of the crude ethanol extract with water-saturated *n*-butanol, the aqueous fraction and the *n*-butanol extract were obtained. The aqueous fraction showed a cytokinin activity at a concentration of 0.1 LE/1 and gave the maximum callus growth at 0.5 LE/1 (Table 1). The cytokinin activity of the aqueous fraction at 0.5 LE corresponds to about 10^{-8} mole of zeatin (cf. Fig. 1) and is comparable with about 10^{-5} mole of zeatin ribotide, a water-soluble cytokinin, because zeatin ribotide is about 1000 times less active than zeatin.[11] On the other hand, the *n*-butanol fraction gave the maximum callus yield at 0.1 LE/1 and higher concentrations rather reduced the growth-promoting activity.

The crude ethanol extract was tested for its cytokinin activity at concentrations from 0.01 to 5 LE/1 (Fig. 2). The extract stimulated slightly the growth of the tobacco callus at 0.01 LE/1. At 0.1 LE/1, the largest growth promotion was observed. Higher concentrations of the extract were rather inhibitory to the callus growth indicating presence of some inhibitory substances. The callus yields were generally lower through all the concentrations tested, compared with those by zeatin and kinetin (cf. Fig. 1). From the maximum callus yield, it was calculated that the ethanol extract equivalent to 100 ml blanching water (0.1 LE) contained about 10^{-9} mole of zeatin.

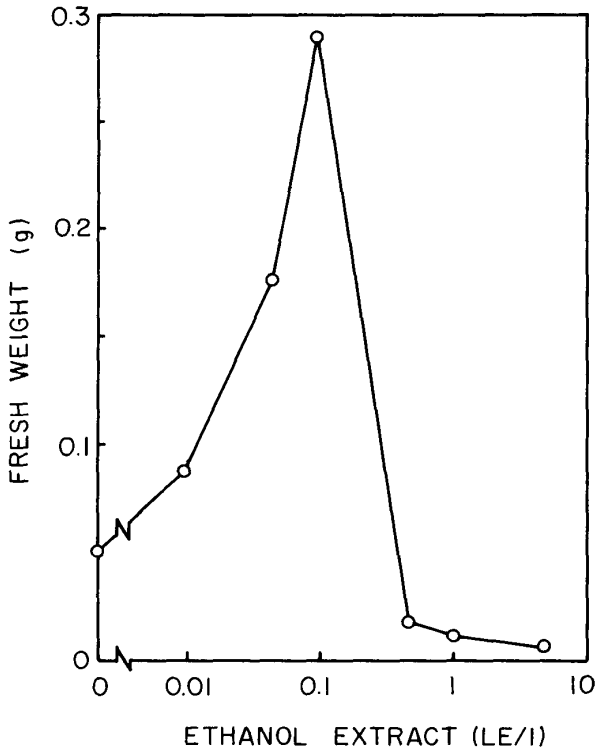

Fig. 2. Effect of the crude ethanol extract obtained from blanching water of bamboo shoots on the growth of the tobacco callus *in vitro*.

To examine additionally water-soluble cytokinins contained in the aqueous fraction, this fraction was further fractionated by a column of Dowex 1. As shown in Table 2, the effluent

fraction was weakly stimulative at 0.05－0.5 LE／1, while the eluate fraction was weakly stimulative at 0.01－0.1 LE／1 and strongly so at 0.5 and 1 LE／1. It can be calculated from Fig. 1 that the eluate fraction of 1 liter blanching water equivalents (1 LE) contained about 2×10^{-8} mole of zeatin. The activity is comparable roughly to that by 2×10^{-5} mole of zeatin ribotide.

Table 1. Cytokinin activity of the aqueous fraction and the *n*-butanol extract tested by the tobacco callus bioassay.

Additives	Conc. (LE／1)*	Fresh weight of callus (g)
—	0	0.04
Aqueous fraction	0.1	0.32
	0.5	0.95
	1	0.80
	5	0.01
n-Butanol extract	0.1	0.49
	0.5	0.20
	1	0.10
	5	0.03

* described in Materials and Methods.

Table 2. Cytokinin activity of the effluent and the eluate after application to a Dowex 1 column.

Additives	Conc. (LE／1)*	Fresh weight of callus (g)
—	0	0.03
Effluent	0.01	0.04
	0.05	0.16
	0.1	0.15
	0.5	0.15
	1	0.02
Eluate	0.01	0.07
	0.05	0.10
	0.1	0.17
	0.5	0.61
	1	1.36

* described in Materials and Methods.

Fractionation of the eluate fraction by a Sephadex LH-20 column was attempted as described in Materials and Methods. Bioassay of all the fractions indicated that the main cytokinin activity came off at 80－120 elution volumes with two peaks and a small activity did at 160-170 ml elution volumes which corresponded to zeatin riboside. The main cytokinin activity was thought to be due to water-soluble cytokinin of larger molecule than zeatin riboside and showed a different elution volume from that of zeatin or its riboside, although it was not clear whether this main cytokinin activity was due to zeatin ribotide or not, because authentic sample of zeatin ribotide could not be available and whether this broad peak was due to one factor or not.

The eluate fraction was studied further by using paper chromatography with three solvent systems in order to characterize the water-soluble cytokinin. Fig. 3-A exhibited evidences of at least two kinds of cytokinins, one is water-soluble cytokinin at Rf 0-0.1 and the other at Rf 0.7-0.8 where zeatin and its riboside located. As authentic sample of zeatin ribotide could not be available, the locus of this cytokinin on the chromatogram was not known. Miller pointed out that a water-soluble cytokinin which was considered to be zeatin ribotide was degraded during experimental procedures and produced zeatin riboside.[18] As zeatin and its riboside are generally eliminated by n-butanol extraction from the aqueous fraction, such cytokinins with high Rf value as zeatin and its riboside observed in the chromatogram as shown in Fig. 3-A are possibly produced by degradation of water-soluble cytokinin with low Rf value.

In the chromatogram developed with water-saturated *sec*-butanol, a similar location of the cytokinin activity to that obtained in the solvent system of water-saturated *n*-butanol was obtained (Fig. 3-B). The water-soluble cytokinin was observed at Rf 0.1-0.3, while another

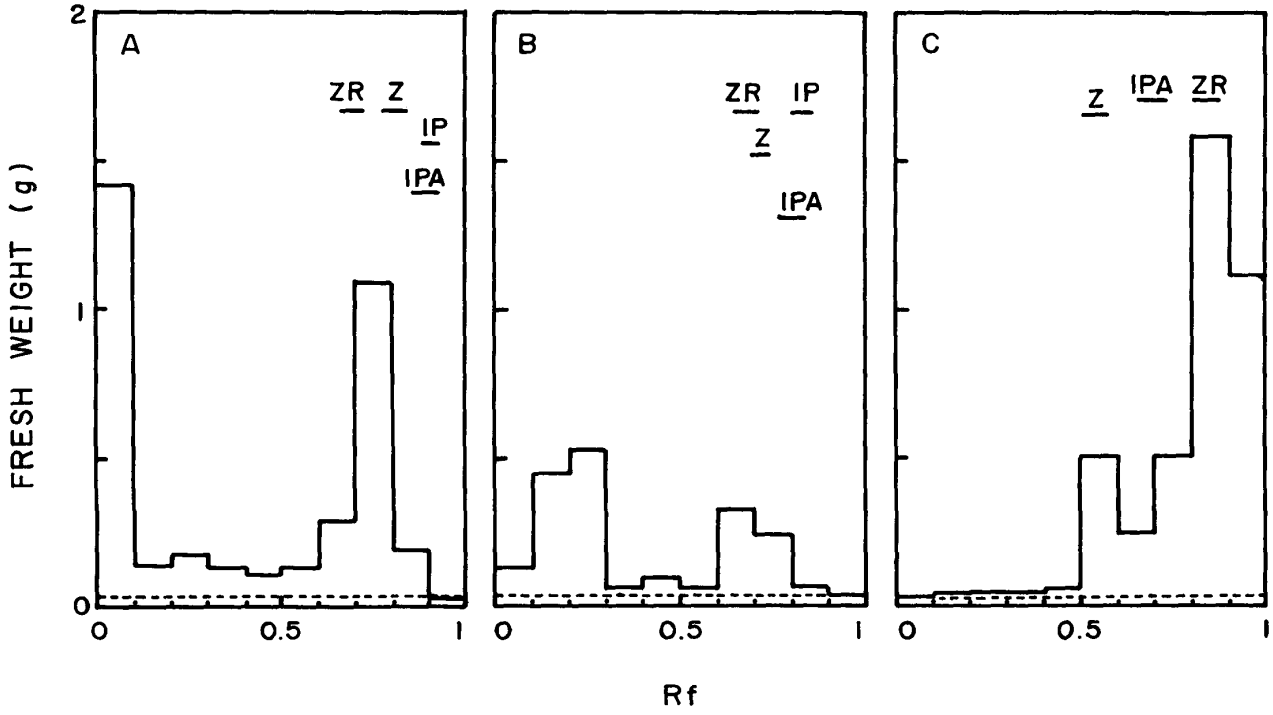

Fig. 3. Location of cytokinin activity in paper chromatograms of the eluate fraction developed with (A) water-saturated *n*-butanol, (B) water-saturated *sec*-butanol and (C) 0.03M boric acid. Lines at the top of histograms indicate the loci of zeatin (Z), zeatin riboside (ZR), N^6-(Δ^2-isopentenyl)adenine (IP) and N^6-(Δ^2-isopentenyl)adenosine (IPA) co-chromatographed. The broken lines represent the fresh weights of the controls without cytokinin.

peak of the activity was observed at Rf 0.6-0.8 where zeatin and its riboside located. In the chromatogram developed with 0.03 M boric acid (Fig. 3-C), cytokinin activity at Rf 0.5-0.6 corresponded to zeatin and that observed at Rf 0.8−0.9 did to zeatin riboside. In this solvent system, water-soluble cytokinins are expected to locate at Rf 0.9-1.0.

Cytokinin ribotides are generally precipitated by barium ions as reported previously.[18] In the present study, it was attempted to precipitate the water-soluble cytokinin in the eluate fraction by barium acetate. As shown in Table 3, cytokinin activity was detected both in the supernatant and the barium precipitate fractions. The cytokinin activity in the supernatant may be due to zeatin or its riboside which perhaps was produced from zeatin ribotide. On the other hand, the barium precipitate showed a high cytokinin activity at a considerably high concentration, 5 LE/l. This might be due to zeatin ribotide which was about 1000 times less active than zeatin.

Barium precipitate was developed with water-saturated *n*-butanol in paper chromatography, and the cytokinin activity was found at Rf 0.-0.1, indicating that the water-soluble cytokinin is precipitated by barium ions and is likely to cytokinin ribotide.

tRNA was hydrolyzed by 0.1 N HCl at 100℃ for 30 minutes and released free base cytokinins.[33] In this study, HCl hydrolysis of cytokinins in the eluate fraction was carried out The water-soluble cytokinins obtained from Rf 0-0.3 zone of paper chromatogram of the eluate fraction developed with water-saturated *n*-butanol was hydrolyzed with 0.1 N HCl. Bioassay revealed that the water-soluble cytokinins were destroyed by this hydrolysis.

The water-soluble cytokinin obtained from Rf 0−0.3 zone of a paper chromatogram of the

Teble 3. Cytokinin activity of the supernatant and the barium ion precipitate tested in the tobacco callus bioassay.

Additives	Conc. (LE/1)*	Fresh weight of callus (g)
—	0	0.02
Supernatant	0.01	0.13
	0.05	0.18
	0.1	0.23
	0.5	0.51
	1	0.30
	5	0.09
Barium precipitate	0.01	0.11
	0.05	0.10
	0.1	0.15
	0.5	0.21
	1	0.15
	5	1.10

* described in Materials and Methods.

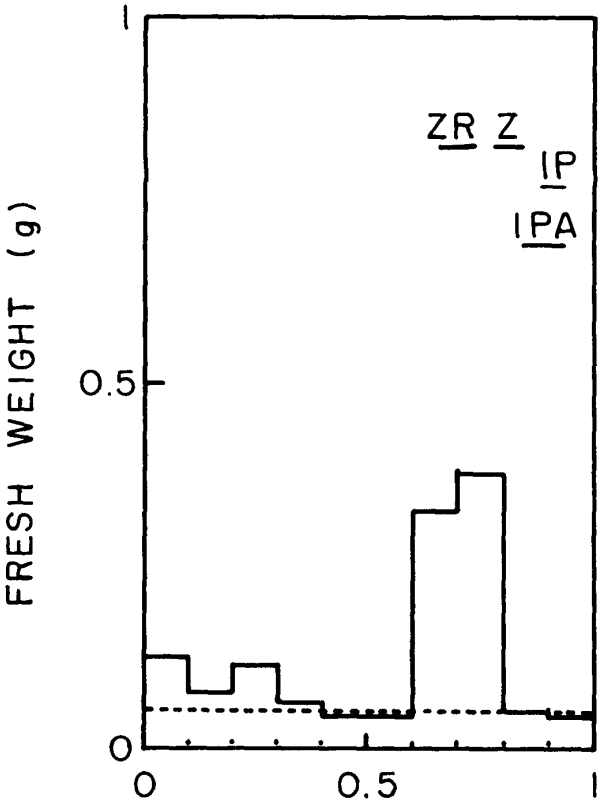

Fig. 4. Location of cytokinin activity in paper chromatogram of the enzymatic hydrolysate developed with water-saturated *n*-butanol. Lines at the top of the histogram indicate the loci of zeatin (Z), zeatin riboside (ZR), N^6-(Δ^2-isopentenyl)adenine (IP) and N^6-(Δ^2-isopentenyl)adenosine (IPA) co-chromatographed.

eluate fraction developed with water-saturated *n*-butanol was hydrolyzed by chicken intestine alkaline phosphatase as described in Materials and Methods. The hydrolysate was developed again with water-saturated *n*-butanol in paper chromatography, and the location of the cytokinin activty on the paper chromatogram was examined.

Fig. 4 indicates that no cytokinin activity was detected at Rf 0-0.3, while a cytokinin activity was observed at Rf 0.6-0.8, where zeatin riboside located. At the loci of N^6-(Δ^2-isopentenyl) adenine and its riboside no cytokinin activity was found. This indicates that the water-soluble cytokinin was hydrolyzed by the alkaline phosphatase and produced a cytokinin which moved with zeatin riboside in paper chromatography. The results thus obtained point out that the water-soluble cytokinin in the blanching water of bamboo shoots is likely to zeatin ribotide. Conclusions as to the chemical structure of the active material must await its isolation in pure form.

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要　約

サイトカイニン活性をもつ多くのプリン誘導体は，人間の細胞培養において抗腫瘍作用など重要な生理作用を持っていることが知られている．一方，このような物質は種々の食品材料植物に，サイトカイニンあるいは tRNA の微量塩基として含まれていることが報告されている．

本研究では，たけのこの煮汁に含まれているサイトカイニンについて，*n*-ブタノール分画，イオン交換樹脂カラム，セファデクスカラムを用いて分画し，植物培養細胞の増殖を生物検定として調べた。その結果，たけのこの煮汁には数種のサイトカイニンが含まれていることがわかった。そのうちの水溶性サイトカイニンを，さらにペーパークロマトグラフィー，バリウム沈殿，酵素分解などによって調べた結果，たけのこの煮汁に含まれている水溶性サイトカイニンはゼアチンのリボチドであると考えられる。煮汁には約 20 μM のゼアチンリボチドに匹敵する活性が含まれていることがわかった．