CYTOKININ BIOSYNTHESIS IN A CELL-FREE SYSTEM FROM CYTOKININ-AUTOTROPHIC TOBACCO TISSUE CULTURES

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1. Introduction

Cytokinins are a group of plant hormones which regulate cell division and differentiation. Cytokinin bases occur in tRNA of microorganisms, animals and plants, but free cytokinins have been found mostly in higher plants [1–4]. Recent studies [5–7] indicate that free cytokinins can arise by biosynthesis de novo in higher plants, but cell-free biosynthesis of free cytokinins in higher plants has not been reported. In the cellular slime mold *Dictyostelium discoideum* a crude enzyme system was isolated to catalyze the synthesis of cytokinin nucleotide [8].

This paper reports the isolation and partial purification of an enzyme system which catalyzes the formation of i^{6}Ado-5′-P from Δ^2-iPP and 5′-AMP from cytokinin-autotrophic tobacco tissue cultures. Small quantities of i^{6}Ado and i^{6}Ade were also detected in the reaction products when less purified enzyme which contained several other enzymes was employed to catalyze the reaction.

2. Materials and methods

2.1. *Chemicals and tissue cultures*

[1-^{14}C]Isopentenyl pyrophosphate (57 μCi/μmol) was obtained from Amersham Corp.; [8-^{14}C]adenosine-5′-phosphate (15 Ci/mmol) was from Schwartz/Mann; Ado, 5′-AMP, i^{6}Ado, i^{6}Ade, 5′-nucleotidase (*Crotalus adamteus* venom) and 3′-nucleotidase (Rye grass) were from Sigma Chem. Co.; i^{6}Ado-5′-P was from PL Biochem. Cytokinin-autotrophic tobacco pith tissue culture was grown on a medium without cytokinin and auxin [9].

Abbreviations: Ado, adenosine; i^{6}Ade, N^6-(Δ^2-isopentenyl)-adenine; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine; i^{6}Ado-5′-P, N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate; Δ^2-iPP or Δ^3-iPP, Δ^2- or Δ^3-isopentenylpyrophosphate, respectively; isomerase, isopentenylpyrophosphate Δ^3–Δ^2-isomerase; Δ^2-isopentenyltransferase, Δ^2-isopentenylpyrophosphate : AMP-Δ^2-isopentenyltransferase

2.2. *Analytical techniques*

Biosynthesized cytokinins were separated by Dowez 1-X4, Cl-form, and Sephadex LH-20 columns followed by paper electrophoresis (Gelman Deluxe TLE Cell, Whatman no. 1 MM paper) with 0.05 M Tris–citrate buffer (pH 3.5) at 22–25°C for 3 h (8.30 V/cm). The biosynthesized cytokinins were partially characterized by Sephadex LH-20 columns, paper electrophoresis and paper chromatography (Whatman no. 1 MM) in a descending fashion in the following solvent systems (v/v): (A) 1-propanol-concentrated NH_4OH–H_2O (60:20:20); (B) 1.4 M LiCl; (C) 95% ethanol–0.1 M $(NH_4)_3BO_3$ (pH 9.0) (1:9); (D) ethyl acetate–1-propanol–water (4:1:2); (E) 2-propanol–water-concentrated NH_4OH (7:2:1).

Chromatograms were cut into 1 cm sections and placed in vials containing scintillation fluid [10]. For liquid samples, an aliquot of <0.5 ml was added to 10 ml Bray's solution [11]. Radioactivity was measured in a Nuclear-Chicago Unilux II scintillation system. A Cary model 14 spectrophotometer was used to measure the quantity of purine derivatives and cytokinins.

2.3. *Enzyme assays*

Isomerase activity was measured by the method in

[12] or by the paper chromatography method [13]. The activity of Δ^2-isopentenyltransferase was assayed by the paper electrophoretic method or by paper chromatography in solvent system A. The first method depended on direct measurements of radioactivity of reaction products after paper electrophoretic separation of radioactive Δ^2-iPP or Δ^3-iPP from i^6Ado-5′-P. The second method was based on the chromatographic separation of the product i^6Ado-5′-P (R_F 0.60) from the substrate Δ^2-iPP (R_F 0.35) or Δ^3-iPP (R_F 0.45) in the solvent system. This solvent system also separated i^6Ado-5′-P from 5′-AMP (R_F 0.20). Unless otherwise specified, the reaction mixture (0.1 ml) contained 100 nCi [1-^{14}C]Δ^3-iPP, 7 mM Tris–HCl buffer (pH 7.0), 20 mM $MgCl_2$, 35 mM $MnCl_2$, 3.5 mM 2-mercaptoethanol, 7 mM KF and isomerase (40 μg protein). The reaction was started by adding the enzyme to the reaction mixture which had been warmed to 37°C. After incubation for 15 min at 37°C, 5′-AMP (final conc. 1 mM) and Δ^2-isopentenyltransferase (5–15 μg protein) were added to the reaction mixture and incubated for an additional 30 min at 37°C. The reaction was stopped by the addition of 1 vol. 95% ethanol. The mixture was then spotted on Whatman no. 1 MM paper and analyzed by paper electrophoretic method, or paper chromatographic method in solvent A. Phosphatase activity was assayed as in [14].

2.4. *Preparation of enzymes*

Isomerase was purified from wheat germ (*Triticum aestivum*) using the method [15]. The purified enzyme had spec. act. 3–5 units/mg protein. One unit of enzyme was defined as the amount that gave a V value for the formation of 1 nmol/min of Δ^2-iPP.

Δ^2-isopentenyltransferase was isolated and partially purified from cytokinin-autotrophic tobacco callus. Enzyme preparation was carried out at 2–4°C. The tobacco callus (55-day old, 100 g) frozen with liquid N_2 was homogenized in a Waring Blendor in 5 vol. wt of buffer A which contained 10 mM Tris–HCl buffer (pH 7.0), 10 mM magnesium acetate, 6 mM KCl, 1 mM EDTA, 6 mM 2-mercaptoethanol, and 25% glycerol. Polyvinyl polypyrrolidone (25 g) was included in the homogenation. The homogenate was centrifuged for 15 min at 10 000 × g. The supernatant was precipitated by 25% ammonium sulfate saturation and centrifuged at 20 000 × g for 20 min. The resulting supernatant was again adjusted to 90% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 20 000 × g for 20 min. The precipitate dissolved in 30 ml buffer A, was dialyzed against 1 l of the same buffer twice. The protein solution (8.5 ml, 12.4 mg protein) was layered onto a Sephadex G-100 column (2.5 × 18 cm) pre-equilibrated with buffer B, that contained 10 mM Tris–HCl (pH 7.0), 1 mM magnesium acetate, 0.6 mM KCl, 0.1 mM EDTA, 0.6 mM 2-mercaptoethanol and 5% glycerol. The protein was eluted with the same buffer solution, and the fractions were analyzed for isomerase and Δ^2-isopentenyltransferase activities as well as protein content. The isomerase was stable for ≥1 month at −70°C but the Δ^2-isopentenyltransferase lost ~90% of its activity after 3 weeks storage at such temperature.

3. Results

3.1. *Partial purification of Δ^2-isopentenyltransferase*

The tobacco tissue extract after ammonium sulfate precipitations was subjected to Sephadex G-100 column chromatography. The protein was eluted with buffer B. Δ^2-isopentenyltransferase activity was generally eluted at 0.63–0.91 bed vol. with peak activity appearing at ~0.79 bed vol. (fig.1). The maximal peak area was pooled (fig.1, pool I) and used in all assays. The degree of purification was ~40-fold when compared to the crude cell extract before ammonium sulfate precipitation. This enzyme preparation contained phosphatase activity which amounted to ~4–6% of that of the specific activity of Δ^2-isopentenyltransferase.

3.2. *Separation and identification of biosynthetic products*

Larger quantities of reaction products were obtained by scaling up of the assay experiments. The reaction products were initially chromatographed on Dowex 1-X4 columns by discontinuous stepwise elution techniques with increasing concentrations of KCl as the eluent. Figure 2B shows a separation pattern of the reaction products. The results were compared with the elution profiles of boiled enzyme plus substrates (result not shown), or authentic i^6Ado-5′-P and of isomerase treated Δ^3-iPP control

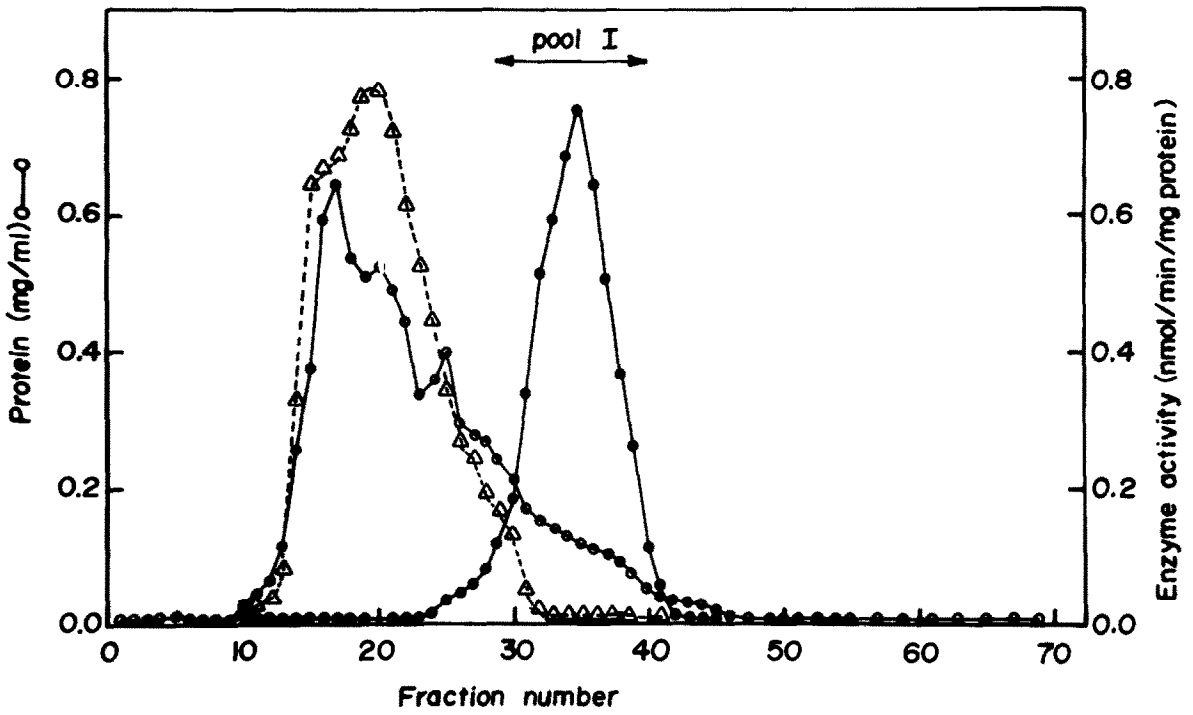

Fig.1. Purification of cytokinin-autotrophic tobacco tissue Δ^2-isopentenyltransferase by Sephadex G-100 column filtration. Protein solution (8.5 ml, 12.4 mg protein fractionated by 25% and 90% ammonium sulfate) was applied onto a column (2.5 × 18 cm) pre-equilibrated with buffer B (10 mM Tris–HCl (pH 7.0), 1 mM magnesium acetate, 0.6 mM KCl, 0.1 mM EDTA, 0.6 mM 2-mercaptoethanol and 5% glycerol). Protein was eluted with the same buffer solution. Fractions of 2 ml were collected. Conditions for enzyme activity assays are described in the text. (●-●) Δ^2-isopentenyltransferase activity; (△. .△) isomerase activity. Fractions indicated by pool I were combined and used to study the characteristics of enzyme activity.

columns (fig.2A). Δ^3-IPP contained ~95% of the pyrophosphate and 5% of the monophosphate according to analysis of paper electrophoresis. An allowance was made in the calculations of substrate concentrations for the contamination of the substrate by the monophosphate. The chromatographic comparison with control samples led to the localization of radioactive i^6Ado-5′-P which clearly separated from the radioactive precursor Δ^2-iPP (Δ^3-iPP reacted with isomerase). The two pooled fractions from Dowex columns (fig.2B, F-a and F-b) were further chromatographed on Sephadex LH-20 columns. The results were compared with relative mobilities of authentic samples (table 1). The relative mobility of F-a sample corresponded to i^6Ado-5′-P, while the F-b sample remains to be identified.

The suspected radioactive cytokinin nucleotide from Sephadex LH-20 columns was analyzed further by paper electrophoresis. The radioactive sample (~1200–1500 cpm) was mixed with unlabeled authentic i^6Ado-5′-P and spotted on Whatman no. 1 MM paper for electrophoresis. The identity of the radioactive sample characterized by the electrophoresis (fig.3A) agreed with the results derived from Sephadex LH-20 column characterization.

To verify further that the radioactive sample was i^6Ado-5′-P, the sample was co-chromatographed on paper with unlabeled i^6Ado-5′-P in 5 solvent systems. The radioactive sample showed one radioactive peak that migrated with the standard marker in these solvent systems. Approximate R_F values for the radioactive sample or unlabeled i^6Ado-5′-P were: 0.60 (solvent system A); 0.78 (solvent system B); 0.89 (solvent system C); 0.19 (solvent system D); and 0.40 (solvent system E).

The evidence strongly suggested that the radio-

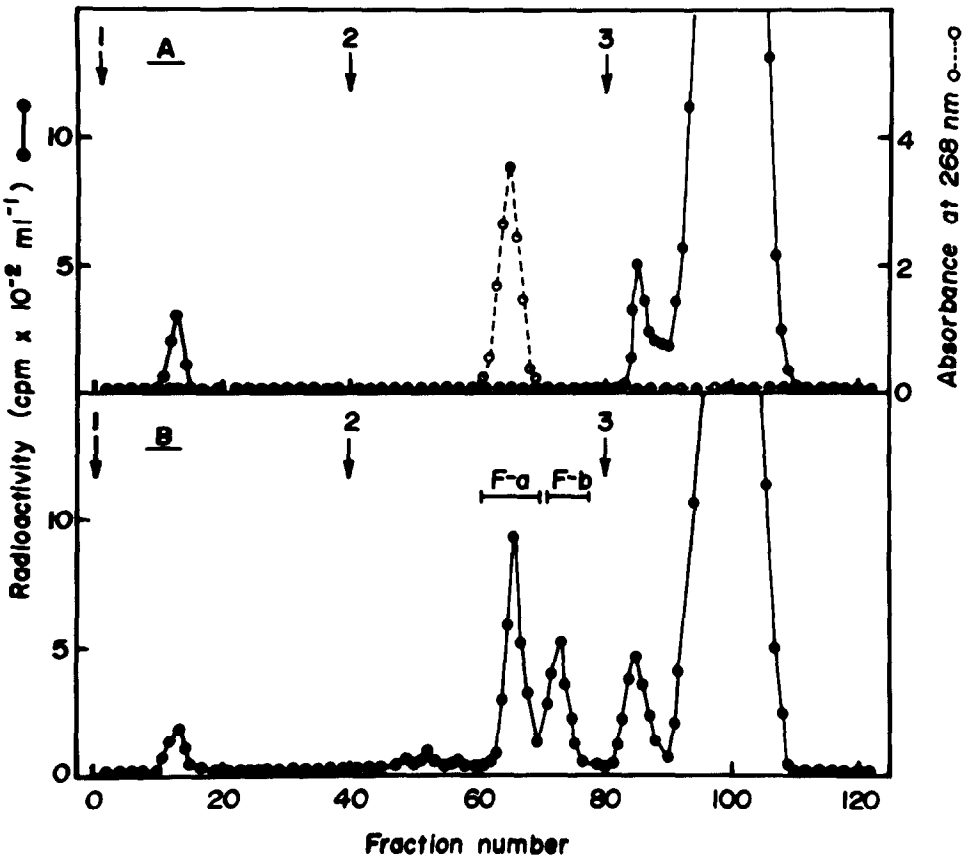

Fig.2. Elution profile of biosynthesized and authentic cytokinin nucleotide on Dowex 1-X4 columns (19.2 × 1.5 cm). (A) Isomerase-treated [1-^{14}C]Δ^3-iPP and unlabelled i^{6}Ado-5′-P. (B) The reaction products of Ado-5′-P and [1-^{14}C]Δ^3-iPP in the presence of isomerase and Δ^2-isopentenyltransferase. Fractions of 3.5 ml were collected. For details see section 2.3. The columns were eluted stepwisely with alkaline solutions adjusted to pH 11.4 with 1 N NaOH : H_2O (arrow 1), 0.1 M KCl (arrow 2), and 0.3 M KCl (arrow 3).

Table 1
Relative mobilities of the precursors and the products of cytokinin biosynthesis on Sephadex LH-20 columns

Compound	Relative mobility[a]
Ade	1.36
Ado	1.21
i^{6}Ade	1.85
i^{6}Ado	1.67
i^{6}Ado-5′-P	0.55
Ado-5′-P	0.62
Δ^3-iPP	0.60
Δ^2-iPP	0.42[b]

[a] The value 1.0 represents an elution volume equivalent to one column volume. The values given are compiled from several different columns

[b] Δ^2-iPP was obtained from Δ^3-iPP in the presence of isopentenylpyrophosphate-Δ^3-Δ^2-isomerase

active product was a cytokinin nucleotide, and it remained to be determined whether the product was the 5′-monophosphate or the 3′-monophosphate. The presumed cytokinin nucleotide was digested with 1 unit (0.05 mg protein) of 5′-nucleotidase of *C. adamanteus* venom in 0.05 M Tris–HCl buffer (pH 7.5) at 37°C for 30 min, and the reaction products were separated by electrophoresis. After the treatment, the metabolite migrated with the corresponding unlabeled i^{6}Ado marker (fig.3B). The experimental evidence indicated that the nucleotide was indeed the 5′-monophosphate.

i^{6}Ado undergoes a unique reaction in the presence of HCl [16]; therefore, as a final confirmation of the identity of the radioactive nucleotide, the radioactive sample was digested with 5′-nucleotidase to form radioactive nucleoside. The radioactive sample was

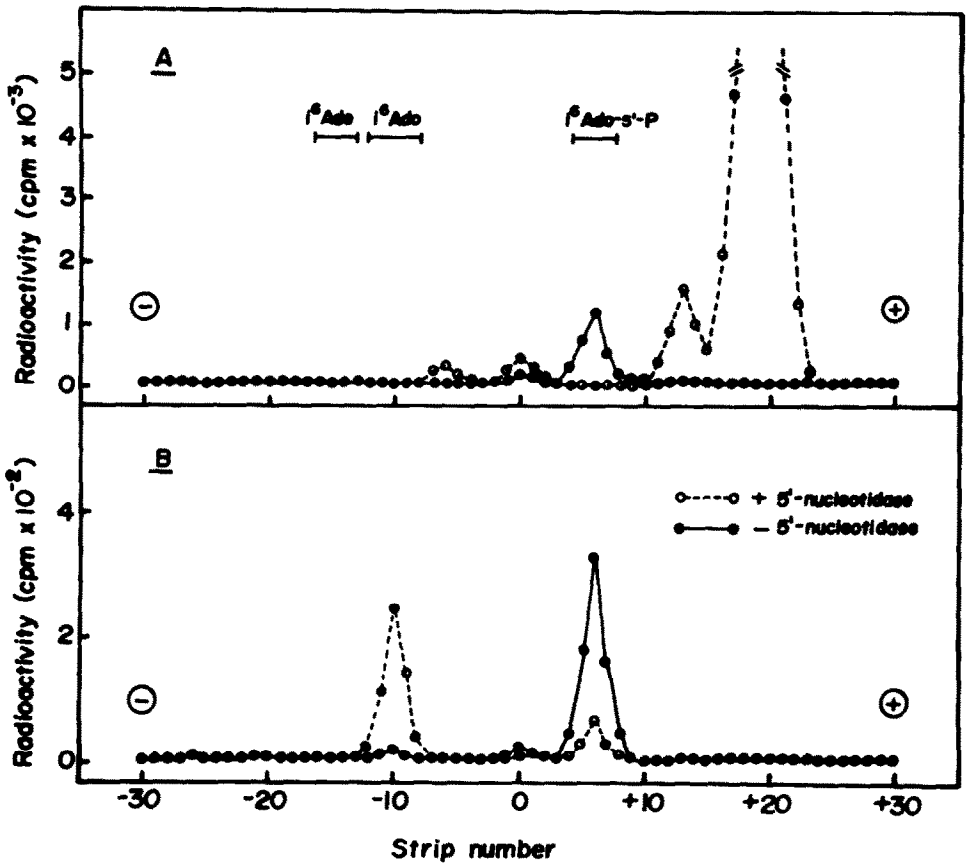

Fig.3. Characterization of biosynthesized cytokinin nucleotide by paper electrophoresis. (A) Electrophoretic analysis of radioactive cytokinin nucleotide (•-•), which was initially isolated from Dowex 1-X4 columns (fig.2B, F-a) and purified further by Sephadex LH-20 columns, and isomerase treated [1-^{14}C]Δ^3-iPP (o. .o). (B) 5′-nucleotidase treatment of the presumed [^{14}C]i^{6}Ado-5′-P. The ^{14}C-labeled peak corresponding to i^{6}Ado-5′-P on the electropherogram (A) was cut and eluted. A portion of the eluate (~1200 cpm) was incubated with (o– –o) and without (•——•) 5′-nucleotidase.

mixed with 3.0 A_{269} units of unlabeled i^{6}Ado and this mixture was hydrolyzed with 200 µl 1 N HCl in a sealed tube at 100°C for 15 min. The reaction was chromatographed on Whatman no. 1 MM paper in solvent system D. The 2 radioactive spots migrated coincidently with the 2 major hydrolytic products, N^6-(3-hydroxy-3-methylbutyl)adenine and 3H-7,7-dimethyl-7,8,9-trihydropyrimido[2,1-i]purine. These results show that the biosynthesized radioactive nucleotide is indeed i^{6}Ado-5′-P.

If crude enzyme preparation, partially purified by ammonium sulfate precipitations, was used as an enzyme source for the Δ^2-isopentenyltransferase to catalyze the cytokinin biosynthesis in the absence of KF, small quantities of i^{6}Ado (10–15%) and i^{6}Ade (5–7%) in addition to i^{6}Ado-5′-P were also produced. The i^{6}Ado may be derived from i^{6}Ado-5′-P by phosphatases and i^{6}Ade from i^{6}Ado by adenosine nucleosidases.

4. Discussion

These results show that cytokinin-autotrophic tobacco callus contains an enzyme system which is capable of catalyzing the synthesis of cytokinin nucleotide from 5′-AMP and Δ^2-iPP. i^{6}Ado and i^{6}Ade were also detected in the reaction products when

crude extracts of the tobacco tissues were used as an enzyme source for the cytokinin biosynthesis. Thus, the free cytokinin nucleosides and bases isolated from cytokinin-autotrophic tobacco callus which have been reported [5,6,17,18] may be derived from the degradation of the cytokinin nucleotides by phosphatases and the nucleosides by nucleosidases in the tissues.

Isopentenylation of Ade, Ado, 5'-AMP or oligoadenylic acids by Δ^2-isopentenylpyrophosphate : tRNA-Δ^2-isopentenyltransferase isolated from *Escherichia coli* [19,20], *Lactobacillus acidophilus* [21] or yeast [22] was reported to be unsuccessful. It remains to be investigated whether the isolated cytokinin biosynthetic enzyme system differs from the enzyme system which catalyzes the isopentenylation of tRNA.

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