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Metabolism of Cytokinin: Deribosylation of Cytokinin Ribonucleoside by Adenosine Nucleosidase from Wheat Germ Cells[1]

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ABSTRACT

Adenosine nucleosidase (adenosine ribohydrolase, EC 3.2.2.7) which catalyzes the deribosylation of N^6-(Δ^2-isopentenyl)adenosine and adenosine to form the corresponding bases was partially purified from wheat germ. This enzyme (molecular weight 59,000 ± 3,000) deribosylates the ribonucleosides at an optimum pH of 4.7. K_m values for the cytokinin nucleoside and adenosine are 2.38 and 1.43 micromolar, respectively, in 50 millimolar Tris-citrate buffer (pH 4.7) at 30 C. The presence of adenosine and other cytokinin nucleosides inhibited the hydrolysis of N^6-(Δ^2-isopentenyl)adenosine but this reaction was insensitive to guanosine, uridine, or 3′-deoxyadenosine. It is hypothesized that an adequate level of "active cytokinin" in plant cells may be provided through the deribosylation of cytokinin riboside in concert with other cytokinin metabolic enzymes.

Cytokinin bases and cytokinin ribonucleosides have been found in various plant cells, and these cytokinins are metabolized in the plant cell to form different metabolites (3, 4, 9, 11, 16, 18). The relative amount of a specific metabolite formed may differ not only from one plant to another, but also in one particular plant or tissue under different physiological conditions. One of the major metabolites formed from a cytokinin ribonucleoside has been reported to be its corresponding base (9, 11, 13). In the *de novo* biosynthesis of cytokinins using a crude enzyme system prepared from plant cells, a cytokinin nucleotide, nucleoside, and base were formed from 5′-AMP and Δ^2-isopentenylpyrophosphate (5). These observations indicate that in plant cells there are enzyme systems catalyzing the formation of cytokinin base from its nucleoside, which in turn can be formed from the corresponding nucleotide (7). Although hydrolytic conversion of Ado[2] to Ade by adenosine nucleosidase (adenosine ribohydrolase, EC 3.2.2.7) has been shown to occur in plant (10, 14, 17) and microbial (20) cells, the role of this enzyme in cytokinin metabolism has not been defined.

We describe here the partial purification of adenosine nucleosidase from wheat germ, the properties of the enzyme, and the kinetics of the deribosylation of cytokinin ribonucleoside by this enzyme system.

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[2] Abbreviations: Ado, adenosine; Ade, adenine; i^6Ado, N^6-(Δ^2-isopentenyl)adenosine; i^6Ade, N^6-(Δ^2-isopentenyl)adenine; *t*-io^{6}Ado, *trans*-6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine.

MATERIALS AND METHODS

Materials. Common nucleic acid bases, nucleosides, wheat (*Triticum sativum*) germ, iodoacetate and *p*-chloromercuribenzoate were obtained from Sigma; cytokinin bases and cytokinin nucleosides were from Calbiochem; [8-^{14}C]Ado (5.9 mCi/mmol) was from Amersham-Searle Corporation. The preparation of [8-^{14}C]i^6Ado (5 Ci/mol) was as described (4).

Analytical Technique. A Cary model 14 spectrophotometer was used to quantify cytokinins, purine bases, and nucleosides. Deribosylated radioactive cytokinins or purine bases were separated from the corresponding nucleosides by thin layer (Polygram CEL 300 UV 254) and/or paper (Whatman 3MM) chromatography with a solvent system (v/v) consisting of 95% ethanol:100 mM $(NH_4)_3BO_3$ (pH 9.0) (1:9). Radioactivity was measured in a Tracor Analytic Mark III liquid scintillation system. For liquid samples, an aliquot of no more than 0.5 ml was added to 10 ml of Bray's solution (2). Protein concentration was determined according to the method of Bradford (1) with BSA as a standard. Molecular weight of the partially purified enzyme was analyzed by Sephadex G-200 column.

Extraction and Fractionation of Enzyme. Wheat germ (135 g) frozen with liquid N_2 was homogenized in a Waring Blendor in 10 mM Tris-HCl buffer (pH 7.0) (4 volumes/weight). The homogenate was filtered through double layers of cheesecloth. The filtrate was centrifuged for 10 min at 10,000*g* and the resulting supernatant was centrifuged again for 25 min at 20,000*g*. The supernatant is referred to as crude extract. The following steps were employed to further purify the extract:

Step 1: Low pH Fractionation. The extract was brought to 30 C and its pH was adjusted to 4.4 over a period of 2 min by adding 1.0 M acetate buffer (pH 4.0). After a further incubation for 5 min at 30 C, the mixture was cooled to 4 C, and the supernatant was collected by centrifugation at 30,000*g* for 10 min.

Step 2: Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to the collected supernatant to 90% saturation over a period of 20 min. The pH was maintained at 4.6 to 4.7 by addition of 1.0 N KOH. After 60 min, the precipitate was collected by centrifugation at 20,000*g* for 30 min. The precipitate was dissolved in 50 ml of 10 mM acetate buffer (pH 4.5) and was stored at 4 C for 16 h. A brown precipitate, formed during the storage, was removed by centrifugation at 10,000*g* for 25 min. The protein solution was dialyzed against 2 liters 10 mM Tris-acetate buffer (pH 4.7) for 16 h and reduced to 10 ml with Carbowax.

Step 3: DEAE-Cellulose Chromatography. The concentrated protein solution (408 mg protein) was applied onto a DEAE-cellulose (Whatman DE-23) column (2.5 × 24 cm) equilibrated with 10 mM Tris-acetate buffer (pH 4.7). The column was eluted with 2.0 bed volumes of this buffer followed by a linear gradient

Table I. *Purification of Adenosine Nucleosidase*

The starting material was 135 g wheat germ.

Procedure	Vol.	Concn.	Total Activity	Protein	Specific Activity[a]	Yield	Purification
	ml	*units/ml*	*units*	*mg/ml*	*units/mg*	*%*	*-fold*
Crude extract	240	0.14	33.6	8.8	0.016	100	(1.0)
pH 4.4 treatment	250	0.14	35	3.8	0.037	104	2.3
90% saturated $(NH_4)_2SO_4$	30	0.85	25.5	13.6	0.063	76	3.9
Precipitate dialyzed DEAE-cellulose	22	0.48	10.6	1.96	0.245	32	15.3
Sephadex G-100	14	0.53	7.42	0.72	0.736	22	46.0

[a] μmol Ade released/min·mg protein at 30 C.

of NaCl (0 to 1.0 M, total volume 330 ml) in the same buffer. The adenosine nucleosidase fractions were pooled and again concentrated with Carbowax.

Step 4: Sephadex G-100 Chromatography. The enzyme solution (43 mg protein in 7 ml) was filtered through a Sephadex G-100 column (2.5 × 34 cm) equilibrated with 10 mM Tris-acetate buffer (pH 4.7). The column was eluted with the same buffer. The fractions containing adenosine nucleosidase activity were pooled and stored at −20 C.

Enzyme Assays. The adenosine nucleosidase was assayed in the following incubation mixture: 2.6 μmol Tris-citrate buffer (pH 4.7), 2.2 nmol [8-^{14}C]Ado (28,600 dpm), or 5.1 nmol [8-^{14}C]i^{6}Ado (56,600 dpm) and the enzyme preparation in a total volume of 120 μl. In control tubes, active enzyme was replaced by a boiled preparation. After 30 min incubation at 30 C, the reaction was terminated by the addition of equal volume of 95% ethanol. To the reaction mixture, 10 μl of 20 mM unlabeled Ade or i^{6}Ade was added as a marker. The mixture was applied to a Polygram CEL 300 UV 254 sheet. The chromatogram was developed with 95% ethanol: 100 mM $(NH_4)_3BO_3$ (pH 9) (1:9, v/v). The strip containing radioactivity was cut out and counted in the scintillation counter.

The activity of adenosine deaminase was determined by the method described previously (4). One unit of the enzyme is defined as the amount of enzyme which produces 1 μmol adenine/min under the assay conditions.

RESULTS

Purification of Adenosine Nucleosidase. The isolation of this enzyme is summarized in Table I. Low pH (4.4) treatment of the crude extract resulted in 2.3-fold purification. These results also demonstrate that this enzyme is stable at acid pH. The enzyme preparation was further fractionated by 90% saturated $(NH_4)_2SO_4$ and separated on a DEAE-cellulose column. The activity of adenosine nucleosidase appeared as a distinct peak in the area of 0.2 to 0.35 M NaCl eluent (Fig. 1). To examine if there is a separate nucleoside hydrase which specifically cleaves the glycosidic bond of cytokinin nucleosidase, i^{6}Ado was used separately as a substrate for enzyme assay in various fractions prior to, as well as after, the enzyme extract was applied to the DEAE-cellulose column. No separate hydrase specific for i^{6}Ado was detected. The active enzyme fractions were pooled and further purified by Sephadex G-100 chromatography. Adenosine nucleosidase activity was generally eluted between 0.75 and 0.95 bed volumes, with peak activity at about 0.84 bed volume (Fig. 2). The activity peak area (Fig. 2, pool I) was pooled and used in all assays. The degree of purification (0.736 units/mg protein) was approximately 46-fold when compared to the crude extract. No adenosine deaminase activity was detected in the pooled fractions. The purified enzyme retained 95% of its activity after 30 days at −20 C.

An apparent mol wt of 59,000 ± 3,000 for the partially purified enzyme was determined by gel filtration on Sephadex G-200 column (2.5 × 28 cm). The molecular weight was calculated from standard marker proteins of known mol wt: myoglobin, 17,000; bovine plasma albumin, 66,000; and ovalbumin, 43,500.

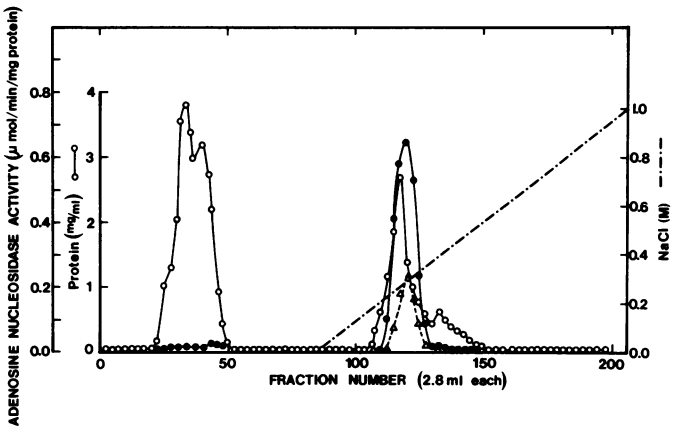

FIG. 1. DEAE-cellulose column chromatography of adenosine nucleosidase from wheat germ cells. Protein solution (9.7 ml, 408 mg protein fractionated by pH 4.4 treatment and by 90% saturated ammonium sulfate) was applied to a column (2.5 × 24 cm) equilibrated with 10 mM Tris-acetate (pH 4.7). The column was eluted with 238 ml of the same buffer followed by a linear gradient of 0 to 1.0 M NaCl. One hundred μl of each fraction were used to measure enzyme activities. Conditions for enzyme activity assays are described in the text. Substrates used were adenosine (●——●) and i^{6}Ado (○——○).

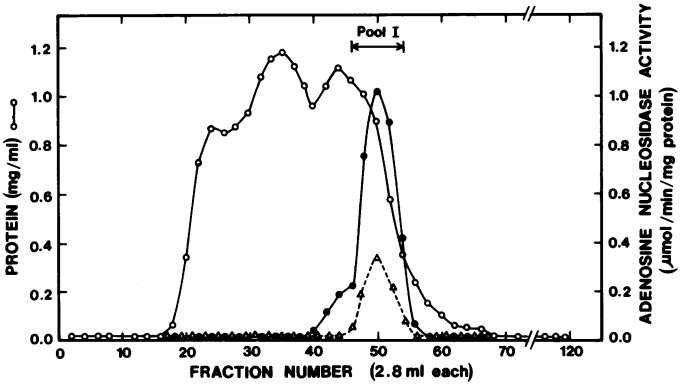

FIG. 2. Purification of wheat germ adenosine nucleosidase by Sephadex G-100 column filtration. The pooled active fractions of adenosine nucleosidase (43 mg protein in 7 ml) obtained from DEAE-cellulose chromatography was applied onto a Sephadex G-100 column (2.5 × 34 cm) equilibrated with 10 mM Tris-acetate buffer (pH 4.7). The column was eluted with the same buffer. Substrates used were adenosine (●——●) and i^{6}Ado (Δ- - -Δ).

Optimum pH. Adenosine nucleosidase had a pH optimum

around 4.7 with either Ado or i^{6}Ado as a substrate; these data were obtained with 50 mM Tris-acetate buffer at pH values from 4 to 5.5. The results are illustrated in Figure 3.

Deribosylation Time Course. The time course studies indicate that the rate of i^{6}Ado or Ado deribosylation by adenosine nucleosidase reached a maximum in 30 min and then leveled off (Fig. 4, only the data of i^{6}Ado deribosylation are shown). The leveling off may be due to end product inhibition, limited presence of the substrate and/or the deterioration of the enzyme in aqueous solution at 30 C. The linearity of i^{6}Ado deribosylation with respect to enzyme concentration is shown in the inset of Figure 4.

Effects of Metal Ions and Sulfhydryl Reagents. The effects of the chloride form of K^+, Mg^{2+}, Ca^{2+}, and Mn^{2+} metal ions on the purified adenosine nucleosidase were studied at 0.5, 1.0, and 10 mM concentrations. No activation or inhibition of the hydrolysis of Ado or i^{6}Ado was detected in the presence of these metal ions. Various concentrations (0.1–10 mM) of sulfhydryl reagents such as iodoacetamide and *p*-chloromercuribenzoate also did not affect the enzyme activity.

Reaction Products. Reaction products were separated by TLC with a 95% ethanol:0.1 M ammonium borate (pH 9.0) (1:9, v/v) solvent system. The solvent system gave a clear separation of Ado or i^{6}Ado from Ade or i^{6}Ade. Approximate R_F values for these compounds were: Ade, 0.36; Ado, 0.64; i^{6}Ade, 0.40 and i^{6}Ado, 0.73. Alternatively, larger quantities of deribosylated nucleoside products were obtained by scaling up of experiments by using ^{14}C-labeled compounds and replacing ^{14}C-labeled substrates with unlabeled ones. The UV absorption spectra of the purified unlabeled deribosylated products were: for i^{6}Ade: max at pH 2, 273 nm; at pH 7, 269 nm; at pH 12, 275 nm; and for Ade: at pH 2, 263 nm; at pH 7, 261 nm; and at pH 12, 268 nm. These values agree with the values for corresponding authentic compounds.

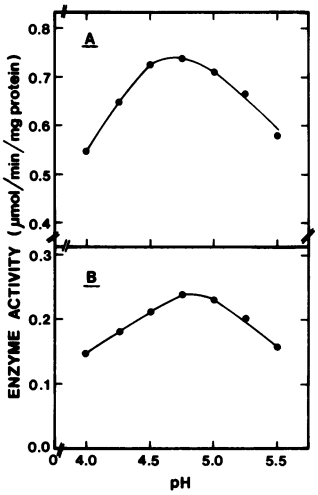

FIG. 3. Effect of pH on Ado (A) and i^{6}Ado (B) deribosylation by adenosine nucleosidase. Standard assay conditions were used, except that the pH of the buffer (50 mM Tris-acetate) was varied.

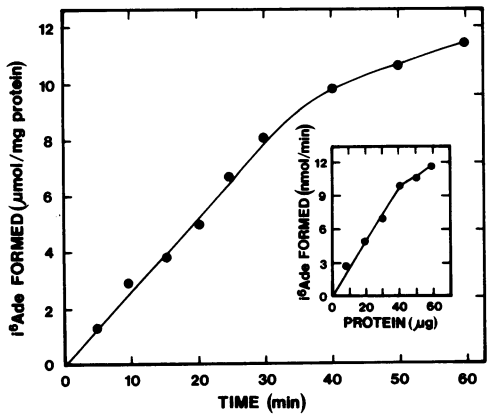

FIG. 4. Time course of i^{6}Ado deribosylation by adenosine nucleosidase. Reactions were carried out under standard assay conditions except incubation times were varied. The effect of protein concentration on i^{6}Ado deribosylation is shown in the inset.

Kinetic Studies. The effects of various cytokinin ribosides and other nucleosides on i^{6}Ado hydrolysis are shown in Table II. The data indicate that the naturally occurring cytokinin riboside *t*-io^{6}Ado had a greater effect on i^{6}Ado hydrolysis than did the synthetic cytokinin ribosides, N^6-furfuryladenosine or N^6-benzyladenosine. The hydrolysis of i^{6}Ado was also affected by Ado, 2′-deoxyadenosine or purine riboside, but not by guanosine, uridine or 3′-deoxyadenosine.

The K_m and V_{max} were calculated from Lineweaver-Burk plots with data from at least eight different substrate concentrations (5×10^{-7} to 1×10^{-4} M) (Table III). At pH 4.7 and 30 C, the K_m values were calculated to be 1.43 and 2.38 μM for Ado and i^{6}Ado, respectively. The V_{max}/K_m ratios indicate that Ado is about 4.5-fold more efficient than i^{6}Ado as a substrate for the adenosine nucleosidase. Addition of 1 mM NaH_2PO_4 did not affect the rate of reaction.

DISCUSSION

The results of these studies demonstrate that i^{6}Ado serves as a substrate for adenosine nucleosidase from wheat germ. Deribo-

Table II. *Effect of Various Nucleosides on i^6 Ado Hydrolysis by Adenosine Nucleosidase*

The purified enzyme (30 μg) was incubated at 30 C for 30 min with 5.1 nmol [8-^{14}C]i^6 Ado (56,000 dpm), 2.6 μmol Tris-citrate buffer (pH 4.7) and indicated amount of other nucleoside in a total volume of 60 μl. In control tubes, active enzyme was replaced by a boiled preparation. [8-^{14}C]i^6 Ade was estimated as described under "Materials and Methods."

Nucleoside Added	Concentration	Relative Activity (i^6 Ade formed)
	mM	%
None		100
3′-Deoxyadenosine	0.5	95
	5.0	96
2′-Deoxyadenosine	0.5	31
	5.0	13
Ado	0.5	22
	5.0	4
N^6-Furfuryladenosine	0.5	47
	5.0	24
io^6 Ado	0.5	30
	5.0	7
N^6-Benzyladenosine	0.5	47
	5.0	23
Guanosine	0.5	100
	5.0	99
Uridine	0.5	99
	5.0	100

Table III. *Kinetic Constants for Adenosine Nucleosidase*

Experimental conditions are as described in Table II, except that the data of K_m and V_{max} were obtained from eight different substrate concentrations.

Compound	K_m	V_{max}	V_{max}:K_m
	μM	*μmol/min·mg protein*	*ratio*
Ado	1.43	0.71	0.50
i^6 Ado	2.38	0.27	0.11

sylation of i^{6}Ado and Ado may be catalyzed by this same enzyme because adenosine nucleosidase was the only enzyme detected capable of cleaving the N-glycosidic bond of these two substrates (Figs. 1 and 2), and these substrates also had the same pH optimum (4.7) for the reaction (Fig. 3). Since the deribosylation takes place in the absence of phosphate and no stimulation occurs in its presence, this enzyme is not a nucleoside phosphorylase.

Kinetic analysis of the reaction indicates that replacement of the N^6-amino group of Ado by an isopentenyl amino side chain decreases the K_m value of the reaction by a factor of about 1.7, and Ado is about 4.5-fold more efficient than i^{6}Ado as a substrate (Table III). The adenosine nucleosidase exhibited a specificity for Ado and N^6-derivatives of Ado, but not for guanosine, 3′-deoxyadenosine, or uridine. Furthermore, the naturally occurring cytokinin, io^{6}Ado, was more effective in inhibiting the deribosylation of i^{6}Ado than were the synthetic cytokinins N^6-benzyladenosine and N^6-furfuryladenosine (Table II).

Although the biological importance of cytokinin nucleoside deribosylation is still obscure, it is well known that in various bioassay systems (18) the cytokinin base is a more active cytokinin than its corresponding riboside. Hecht *et al.* (12), working with various cytokinin-active analogs, indicated that cytokinins can probably function without ribosylation, and that the free base itself might best fit the requirements of the "active" compound. While the question of whether cytokinin base *per se* serves as the "active form" of cytokinin remains to be resolved, cytokinin-binding protein studies (6, 8, 15, 19) suggest that cytokinin base may be one of the active forms of cytokinin. Thus, the enzymic regulation of the interconversion of cytokinin nucleotide, nucleoside and base may be important for an adequate level of "active cytokinin" in plant cells.

LITERATURE CITED

1. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
2. Bray G 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal Biochem 1: 279–285
3. Chen CM, B Petschow 1978 Cytokinin biosynthesis in cultured rootless tobacco plants. Plant Physiol 62: 861–865
4. Chen CM, B Petschow 1978 Metabolism of cytokinin. Ribosylation of cytokinin bases by adenosine phosphorylase from wheat germ. Plant Physiol 62: 817–874
5. Chen CM DK Melitz 1979 Cytokinin biosynthesis in a cell-free system from cytokinin-autotrophic tobacco tissue cultures. FEBS Lett 107: 15–20
6. Chen CM, DK Melitz, B. Petschow, RL Eckert 1980 Isolation of cytokinin-binding protein from plant tissues by affinity chromatography. Eur J Biochem 108: 379–387
7. Chen CM, SM Kristopeit 1981 Metabolism of cytokinin: dephosphorylation of cytokinin ribonucleotide by 5′-nucleotidases from wheat germ cytosol. Plant Physiol 67: 494–498
8. Erion JL, JE Fox 1981 Purification and properties of a protein which binds cytokinin-active 6-substituted purines. Plant Physiol 67: 156–162
9. Fox J 1969 The cytokinins. *In* M Wilkins, ed, The Physiology of Plant Growth and Development. McGraw Hill, London, pp 85–123
10. Guranowski A, Z Schneider 1977 Purification and characterization of adenosine nucleosidase from barley leaves. Biochim Biophys Acta 482: 145–158
11. Hall RH 1970 N^6-(Δ^2-isopentenyl)adenosine: chemical reactions, biosynthesis, metabolism and significance to the structure and function of tRNA. *In* JN Davidson, WE Cohn, eds, Progress in Nucleic Acid Research and Molecular Biology, Vol 10. Academic Press, New York, pp 57–86
12. Hecht SM, RB Frye, D Werner, DS Hawrelak, F Skoog, RY Schmitz 1975 On the "activation" of cytokinins. J Biol Chem 250: 7343–7351
13. Laloue M, C Terrine, J Guern 1977 Cytokinins: metabolism and biological activity of N^6-(Δ^2-isopentenyl)adenosine and N^6-(Δ^2-isopentenyl)adenine in tobacco cells and callus. Plant Physiol 59: 478–483
14. Mazelis M, RK Creveling 1963 An adenosine hydrolase from brussel sprouts. J Biol Chem 238: 3358–3361
15. Moore FH III 1979 A cytokinin binding protein from wheat germ. Isolation by affinity chromatography and properties. Plant Physiol 64: 594–599
16. Parker CW, DS Letham 1973 Regulators of cell division in plant tissues. XVI. Metabolism of zeatin by radish cotyledons and hypocotyls. Planta 114: 199–218
17. Poulton JE, VS Butt 1976 Partial purification and properties of adenosine nucleosidase from leaves of spinach beet (*Beta vulgaris L.*). Planta 131: 179–185
18. Skoog F, DJ Armstrong 1970 Cytokinins. Annu Rev Plant Physiol 21: 359–384
19. Sussman MR, H Kende 1978 *In vitro* cytokinin binding to a particulate fraction of tobacco cells. Planta 140: 251–259
20. Takagi Y, BL Horecker 1957 Purification and properties of a bacterial riboside hydrolase. J Biol Chem 225: 77–87