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Metabolism of Cytokinin

RIBOSYLATION OF CYTOKININ BASES BY ADENOSINE PHOSPHORYLASE FROM WHEAT GERM[1]

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ABSTRACT

As part of the study of cytokinin metabolic pathways, an enzyme, adenosine phosphorylase (EC 2.4.2.-), which catalyzed the ribosylation of N^6-(Δ^2-isopentenyl)adenine, N^6-furfuryladenine, and adenine to form the corresponding nucleosides, was partially purified from wheat (*Triticum aestivum*) germ. The pH optimum for the ribosylation of the cytokinins and adenine was from 6.5 to 7.8; for guanine and hypoxanthine it was from 7.0 to 8.5. At pH 7.2 (63 millimolar N-2-hydroxyethyl piperazine-N'-ethanesulfonic acid) and 37 C the K_m for N^6-(Δ^2-isopentenyl)adenine was 57.1 micromolar; N^6-furfuryladenine, 46.5 micromolar; adenine, 32.2 micromolar; and the V_{max} for N^6-(Δ^2-isopentenyl)adenine, N^6-furfuryladenine, and adenine were 134.7, 137.1, and 193.1 nanomoles per milligram protein per minute, respectively. The equilibrium constants of the phosphorolysis of N^6-(Δ^2-isopentenyl)adenosine and adenosine by this enzyme indicated that the reaction strongly favored nucleoside formation. This enzyme was shown to be distinct from inosine-guanosine phosphorylase based on the differences in the Sephadex G-100 gel filtration behaviors, pH optima, and the product and p-hydroxymercuribenzoate inhibitor studies. These results suggest that adenosine phosphorylase may play a significant role in the regulation of cytokinin metabolism.

The metabolism of cytokinins has been studied in a variety of plant tissues (2–4, 7–9, 11, 13, 15) and some of the enzymes which regulate cytokinin metabolism have also been isolated (1, 6). One of the major metabolites formed from the cytokinin base is the corresponding ribonucleoside. Purine nucleoside phosphorylase (purine nucleoside, orthophosphate ribosyl transferase, EC 2.4.2.1) which catalyzes a reversible phosphorolysis of purine nucleosides is generally believed to be inactive toward Ado[2] (14, 16, 19) and cytokinin nucleoside. One of the possible pathways by which the cytokinin base can be converted to the corresponding ribonucleoside is the phosphoribosylation of the cytokinin base to the ribonucleotide and then conversion to the corresponding ribonucleoside by 5'-nucleotidase (1). Senesi *et al.* (17) recently reported that in *Bacillus subtilis* adenosine phosphorylase (EC 2.4.2.-) is distinct from purine nucleoside phosphorylase and that this enzyme catalyzes the formation of Ado from Ade. Adenosine phosphorylase activity was also found in some species of mycoplasmas (10) and mammalian cells (5).

This paper presents the first direct evidence that in plant cells the ribosylation of cytokinin base and Ade is catalyzed by adenosine phosphorylase, and that this enzyme is distinct from inosine-guanosine phosphorylase. The equilibrium constant of the ribosylation reaction strongly favors i^6Ado or Ado formation. Partial purification of adenosine phosphorylase and characteristics of the enzyme activity are also described.

MATERIALS AND METHODS

Chemicals. Ade, i^6Ade, i^6Ado, N^6-furfuryladenine, N^6-furfuryladenosine, ribose-1-P (dicyclohexylammonium salt), *p*-chloromercuribenzoic acid (Na salt), and HEPES were obtained from Sigma Chemical Company. [8-^{14}C]Ade (50 mCi/mmol), [8-^{14}C]Ado (50 mCi/mmol), and [8-^{14}C]guanine (58 mCi/mmol) were from Schwarz/Mann. [8-^{14}C]Hypoxanthine (62 mCi/mmol) and [8-^{14}C]N^6-furfuryladenine (15 mCi/mmol) from Amersham Searle Corp. The preparation of [8-^{14}C]i^6Ade (5 mCi/mmol) and [8-^{14}C]i^6Ado (5 mCi/mmol) was as described (4).

Analytical Techniques. Ribosylated compounds were separated by paper chromatography (Whatman No. 3MM) using 95% ethanol-0.1 M ammonium borate (pH 9.0) (1:9, v/v) solvent system. Chromatograms were cut into 1-cm sections and placed in vials containing scintillation fluid (4). Radioactivity was measured in a Nuclear-Chicago Unilux II scintillation system. Counting efficiency of paper chromatogram sections was 74% for ^{14}C. A Cary model 14 spectrophotometer was used to measure the quantity of purine base and its corresponding riboside. Protein concentration was determined by the method of Lowry *et al.* (12).

Extraction and Fractionation of Enzymes. The following steps were performed at 2 to 4 C. Wheat germ (*Triticum aestivum*) (30 g) frozen with liquid N_2 was homogenized in a Waring Blendor in 5 volumes/weight of 63 mM HEPES buffer (pH 7.2) containing PVP (3 g). The homogenate was centrifuged for 15 min at 15,000*g* and the resulting supernatant was centrifuged for 20 min at 20,000*g*. The supernatant was then filtered through cheesecloth. The filtrate was precipitated by 55% ammonium sulfate saturation and the resulting precipitate was collected by centrifugation at 20,000*g* for 20 min. The precipitate dissolved in 20 ml of 63 mM HEPES (pH 7.2) contained 953 mg of protein. The protein solution (11 ml) was layered onto a Sephadex G-100 column (2.5 × 30 cm) which had previously been equilibrated with 63 mM HEPES (pH 7.2). The protein was eluted with the same buffer solution, and the fractions were analyzed for adenosine phosphorylase, inosine-guanosine phosphorylase, and adenosine deaminase activities and protein content. The enzyme preparation was stored at −70 C and the adenosine phosphorylase was stable for at least 1 month.

Enzyme Assays. The assay of adenosine phosphorylase was based on the conversion of ^{14}C-labeled purines to the corresponding nucleosides and their estimation after chromatographic separation of the remaining substrate. Unless otherwise specified, the reaction mixture (0.4 ml) contained 16.7 μM of ^{14}C-labeled purine or cytokinin base, 1.3 mM ribose-1-P, 63 mM HEPES (pH 7.2), and the enzyme (80–800 μg protein). The reaction was started by adding the enzyme to the reaction mixture which had been warmed to 37 C. After incubation for 30 min at 37 C, the reaction was stopped by addition of an equal volume of 95% ethanol. The

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[2] Abbreviations: Ade: adenine; Ado: adenosine; i^6Ade: N^6-(Δ^2-isopentenyl)adenine; i^6Ado: N^6-(Δ^2-isopentenyl)adenosine.

mixture was then spotted on Whatman No. 3MM paper for chromatography.

The assay of adenosine deaminase was as previously described (3).

RESULTS

Separation and Identification of Reaction Products. Reaction products were separated by paper chromatography using a 95% ethanol-0.1 M ammonium borate (pH 9.0) (1:9, v/v) solvent system. This solvent system gave a clear separation of each purine ribonucleoside from the corresponding free base in 5 hr at room temperature (22-25 C). Approximate R_F values for the various compounds were: i^{6}Ade, 0.54; i^{6}Ado, 0.76; N^6-furfuryladenine, 0.51; N^6-furfuryladenosine, 0.76; Ade, 0.42; Ado, 0.68; hypoxanthine, 0.61; iosine, 0.80; guanine, 0.31; and guanosine, 0.56.

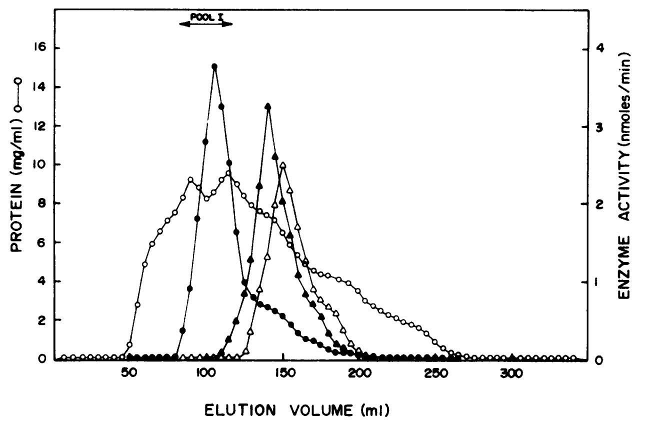

FIG. 1. Purification of wheat germ adenosine phosphorylase by Sephadex G-100 column filtration. Protein solution (11 ml, 953 mg of protein fractionated by 55% ammonium sulfate) was applied onto a column (2.5 × 30 cm) previously equilibrated with a solution of 63 mM HEPES (pH 7.2). Protein was eluted with the same buffer solution. Fractions of 5 ml were collected. One hundred µl of each fraction were used to measure enzyme activities. Conditions for enzyme activity assays are described in the text. (●——●): Adenosine phosphorylase activity; (△——△): inosine-guanosine phosphorylase activity; (▲——▲): adenosine deaminase activity; (○——○): protein. Fractions indicated by pool I were combined and used to study the characteristics of enzyme activity.

Alternatively, larger quantities of ribosylated purine products were obtained by scaling up of experiments using ^{14}C-labeled compounds and replacing ^{14}C-labeled substrates with unlabeled ones. The UV absorption spectra of the purified unlabeled ribosylated products were: i^{6}Ado: λ_{max} at pH 2.0, 265 nm; pH 7-12, 269 nm; N^6-furfuryladenosine: λ_{max} at pH 2.0, 266 nm; pH 7-12, 271 nm; and Ado: λ_{max} at pH 2.0, 257 nm; pH 7.0, 260 nm; pH 12.0, 259 nm. These values are identical to the values of corresponding authentic ribonucleosides.

Partial Purification of Adenosine Phosphorylase. The wheat germ extract was subjected to Sephadex G-100 column chromatography. The protein was eluted with 63 mM HEPES (pH 7.2). Adenosine phosphorylase activity was generally eluted between 0.58 and 0.92 bed volumes, with peak activity appearing at about 0.73 bed volume (Fig. 1). The maximal peak area (Fig. 1, pool I), devoid of inosine-guanosine phosphorylase activity, was pooled and used in all assays. The degree of purification was approximately 23-fold when compared to the crude cell extract. This enzyme preparation contained adenosine deaminase activity which amounted to about 5 to 10% of that of adenosine phosphorylase activity.

Characteristics of Enzyme Activity. The time course studies indicated that the rate of ribosylation reached a maximum in 60 min and then decreased (Fig. 2). The rate of ribonucleoside formation from purine bases was linear with respect to enzyme concentration (data not shown). The chromatogram of each assay mixture, except those containing Ade, showed two major radioactive peaks, one corresponding to the base, the other to the corresponding ribonucleoside. In case of the Ade assays, the chromatogram showed three radioactive peaks, one corresponding to Ade, the other to Ado, and a third small peak in the region of inosine which amounted to about 5 to 10% of the biosynthesized Ado.

The effect of pH on the reaction rate was determined at eight pH values between 4.0 and 9.0 in 63 mM sodium citrate (pH 4-6) and 63 mM HEPES (pH 6-9). There was a broad pH optimum over the range 6.5 to 7.5 for ribosylation of both Ade and i^{6}Ade by adenosine phosphorylase. On the other hand, the pH optimum for ribosylation of guanine or hypoxanthine by inosine-guanosine phosphorylase ranged from 7.0 to 8.5 (data not shown). The adenosine phosphorylase activity present in wheat germ cells is

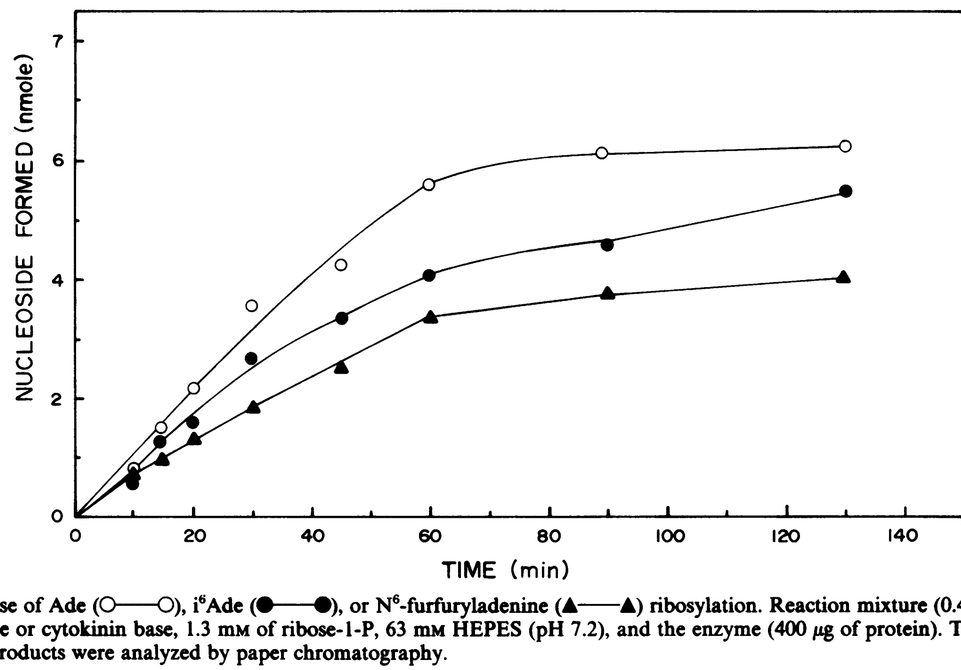

FIG. 2. Time course of Ade (○——○), i^{6}Ade (●——●), or N^6-furfuryladenine (▲——▲) ribosylation. Reaction mixture (0.4 ml) contained 1.67 µM of ^{14}C-labeled adenine or cytokinin base, 1.3 mM of ribose-1-P, 63 mM HEPES (pH 7.2), and the enzyme (400 µg of protein). The reaction was carried out at 37 C and the products were analyzed by paper chromatography.

distinct from inosine-guanosine phosphorylase.

The conversion of purine bases to nucleosides depended upon the addition of ribose-1-P, while the phosphorolysis of nucleosides was dependent on the presence of inorganic phosphate. The K_m and V_{max} were calculated by the method of the Lineweaver-Burk plot with data from at least five different substrate concentrations (Table I). At pH 7.2 and 37 C, the K_m was calculated to be 32.2, 57.1, and 46.5 μM for Ade, i^{6}Ade, and N^6-furfuryladenine, respectively, whereas the V_{max} was 193.1, 134.7, and 137.1 pmol/mg protein·min for Ade, i^{6}Ade, and N^6-furfuryladenine, respectively. Table I shows that the K_m of i^{6}Ade and N^6-furfuryladenine for the adenosine phosphorylase approximates that of Ade.

***p*-Chloromercuribenzoate and Product Inhibition Studies.** If the phosphorolysis of Ado and inosine or guanosine is catalyzed by different enzymes from wheat germ, it should also be possible to distinguish these enzymes by inhibitor studies. Parks and Agarwal (16) reported that *p*-chloromercuribenzoate inhibited several nucleoside phosphorylase preparations. The activity of wheat germ adenosine phosphorylase and inosine-guanosine phosphorylase was inhibited by 30-min initial incubation with *p*-chloromercuribenzoate (Fig. 3). The activity toward hypoxanthine and guanine was more affected than the activity toward Ade and N^6-furfuryladenine. The activity of inhibitor-free controls was not affected by 30-min initial incubation.

The results of the inhibition of the ribosylation of purines by the products of the reaction are shown in Table II. Ado selectively inhibited the ribosylation of Ade, but no significant effect on the ribosylation of guanine or hypoxanthine. On the other hand, inosine and guanosine selectively cause inhibition of ribosylation of hypoxanthine and guanine. These results, together with the results of *p*-chloromercuribenzoate inhibition studies, clearly show that adenosine phosphorylase isolated from wheat germ cells is distinct from inosine-guanosine phosphorylase.

Equilibrium Constant. To study whether the equilibrium of the reaction favors the Ado or i^{6}Ado formation, equilibrium constants were determined. The reaction mixture, containing 63 mM HEPES (pH 7.2), 3 mM K_2HPO_4, 0.5 μM [8-^{14}C]Ado or 0.5 μM [8-^{14}C]i^{6}Ado, and 0.4 mg of adenosine phosphorylase in a final volume of 0.4 ml, was incubated for 1 hr at 37 C. As a control the reaction mixture was also stopped at zero time. The reaction products were assayed by paper chromatography as described under "Materials and Methods." The concentration of [8-^{14}C]Ado or [8-^{14}C]i^{6}Ado determined at zero time under these conditions was identical to that of the original reaction mixture. The equilibrium constant for i^{6}Ado or Ado (represented by i^{6}Ado only in the equation):

$$\mathrm{Keq} = \frac{[\mathrm{i^6Ade}]_{eq}\cdot[\text{ribose-1-P}]_{eq}}{[\mathrm{i^6Ado}]_{eq}\cdot[\mathrm{Pi}]_{eq}}$$

was calculated by assuming $[\mathrm{i^6Ade}]_{eq} = [\text{ribose-1-P}]_{eq} = [\mathrm{i^6Ado}]_{\text{zero time}} - [\mathrm{i^6Ado}]_{eq}$ and $[\mathrm{Pi}]_{eq} = [\mathrm{Pi}]_{\text{zero time}} - [\mathrm{i^6Ade}]_{eq}$.

The average equilibrium constant values from experiments each repeated three times with less than 15% variation were: 1.48×10^{-3} for Ado and 1.38×10^{-3} for i^{6}Ado. Therefore, the equilibrium of the reaction strongly favors Ado or i^{6}Ado formation.

TABLE I

Michaelis Constants and Maximum Initial Velocity for the Ribosylation of Purine Derivatives by Adenosine Phosphorylase[a]

Compound	εmax (×10^{-4})	Km (M×10^{-6})	Vmax (pmol per mg protein·min)	Relative Rate
Ade	1.34	32.2	193.1	100
i^{6}Ade	1.94	57.1	134.7	69.7
N^6-furfuryladenine	1.86	46.5	137.1	71.0

[a]The Km and Vmax values were determined with 63 mM HEPES buffer (pH 7.2). Each incubation mixture contained 1.3 mM ribose 1-phosphate, 200 μg protein and a purine substrate (with data on at least five different substrate concentrations of each purine derivatives).

DISCUSSION

The results of this study show that the ribosylation of i^{6}Ade, or

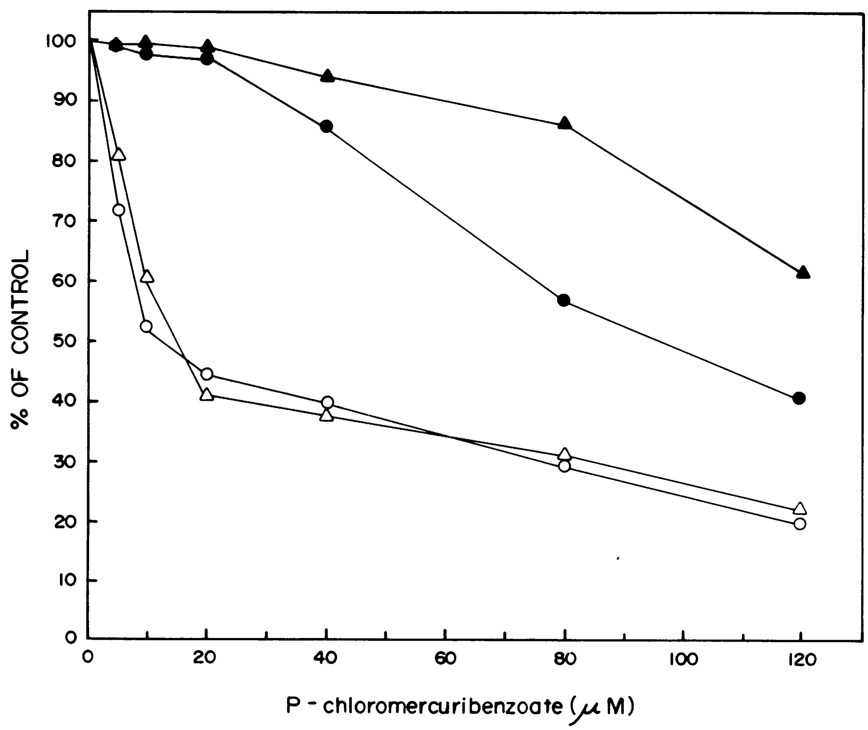

FIG. 3. Inhibition of adenosine phosphorylase and inosine-guanosine phosphoylase by *p*-chloromercuribenzoate. Assay conditions used were as described in Figure 2 except that enzyme preparations and *p*-chloromercuribenzoate were initially incubated for 30 min before the addition of different substrates. (●——●): Ade; (▲——▲): N^6-furfuryladenine; (○——○): guanine; (△——△): hypoxanthine.

TABLE II

Product Inhibition of the Ribosylation of Purines[a]

Enzyme Preparation	Nucleoside Added (1.0 mM)	Percent Inhibition of the Ribosylation		
		Ade	Guanine	Hypoxanthine
Adenosine	0	0	-	-
Phosphorylase	Adenosine	43	-	-
	Guanosine	7	-	-
	Inosine	9	-	-
Inosine-Guanosine	0	-	0	0
Phosphorylase	Adenosine	-	5	7
	Guanosine	-	41	32
	Inosine	-	35	46

[a]The ribosylation of purine was measured in the absence and presence of inhibitors. The reaction mixture (0.4 ml) which contained 1.67 µM ^{14}C-purine, 400 µg protein, 63 mM HEPES buffer (pH 7.2), 1.3 mM ribose 1-phosphate and the inhibitor (1.0 mM ribonucleoside) was incubated for 30 min at 37 C. The data represent the average of 2 separate experiments which did not vary more than 12%. In controls, the purine ribonucleosides formed were about 3,000 to 3,300 cpm.

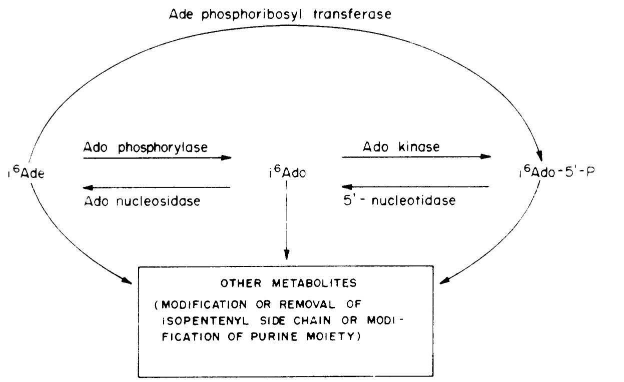

FIG. 4. Possible pathways for interconversion of cytokinin base, ribonucleoside, and ribonucleotide.

other cytokinin bases, is catalyzed by adenosine phosphorylase from wheat germ cells. These results together with previous evidence indicate that there are four major competing enzymic pathways by which i^{6}Ade can be metabolized in plant cells (Fig. 4): (*a*) phosphorylation of i^{6}Ado by adenosine kinase (1); (*b*) ribosylation of i^{6}Ade by adenosine phosphorylase; (*c*) modification or removal of the isopentenyl side chain of i^{6}Ade by crude enzyme preparations (2, 9); and (*d*) modification of purine moiety of cytokinin base by various enzymes (4, 8, 15).

Although inosine-guanosine phosphorylase of several mammalian sources catalyzes a reversible phosphorolysis of Ado, its unfavorable kinetic parameters with respect to those of hypoxanthine and guanosine are against the role of Ade as a physiological substrate (14, 19). The wheat germ adenosine phosphorylase which catalyzes the formation of nucleoside is shown to be distinct from inosine-guanosine phosphorylase by several criteria: (*a*) the two enzyme activities can be clearly separated by ammonium sulfate fractionation followed by Sephadex G-100 gel filtration (Fig. 1); (*b*) treatment of these two enzyme preparations with *p*-chloromercuribenzoate inhibited the two enzyme activities to a different extent (Fig. 3); (*c*) Ado selectively inhibited the ribosylation of Ade catalyzed by adenosine phosphorylase, while inosine and guanosine selectively inhibited the ribosylation of hypoxanthine and guanine catalyzed by inosine-guanosine phosphorylase (Table II); and (*d*) pH *versus* activity curve with Ade as the substrate was markedly different from the curve with hypoxanthine as the substrate.

The equilibrium constant of the reaction catalyzed by adenosine phosphorylase strongly favors cytokinin nucleoside formation. In various plant bioassay systems, cytokinin base has been shown to be more effective than the corresponding cytokinin ribonucleoside in promoting cell growth (18), thus, the physiological role of adenosine phosphorylase in the synthesis of cytokinin ribonucleoside remains to be established. This enzyme, together with other cytokinin metabolic enzymes, may be involved in the regulation of the level of cytokinin available to the plant cells and/or in the control of cytokinin metabolic pathways.

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