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

Chong-Maw Chen and Susan M. Kristopeit
Department of Life Science and Biomedical Research Institute, University of Wisconsin-Parkside, Kenosha, Wisconsin 53141

ABSTRACT

Adenosine nucleosidase (adenosine ribohydrolase, EC 3.2.2.7) which catalyzes the deribosylation of N6-(Δ2-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. Km 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 N6-(Δ2-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 Δ2-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 AdoΔ to Ado 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.

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; [8-14C]Ado (5.9 mCi/mm) was from Amersham-Searle Corporation. The preparation of [8-14C]Ado (5 Ci/mmol) 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 (NH4)2BO3 (pH 9.0) (1:9). Radioactivity was measured in a Tracer 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 N2 was homogenized in a Waring Blender in 10 mM Tris-HCl buffer (pH 7.0) (4 volumes/weight). The homogenerate was filtered through double layers of cheesecloth. The filtrate was centrifuged for 10 min at 10,000g and the resulting supernatant was centrifuged again for 25 min at 20,000g. 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 30,000g 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 M KOH. After 60 min, the precipitate was collected by centrifugation at 20,000g 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 10,000g 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 x 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...
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 [8-14C]Ado (28,600 dpm), or 5.1 nmol [8-14C]i6Ade (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 i6Ade 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 (NH4)2BO3 (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 1. 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 (NH4)2SO4 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 hydase which specifically cleaves the glycosidic bond of cytokinin nucleosidase, [3H]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 hydase specific for [3H]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.

**Optimum pH.** Adenosine nucleosidase had a pH optimum

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**Table 1. Purification of Adenosine Nucleosidase**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol.</th>
<th>Conc.</th>
<th>Total Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>240</td>
<td>0.14</td>
<td>33.6</td>
<td>8.8</td>
<td>0.016</td>
<td>100</td>
<td>(1.0)</td>
</tr>
<tr>
<td>pH 4.4 treatment</td>
<td>250</td>
<td>0.14</td>
<td>35</td>
<td>3.8</td>
<td>0.037</td>
<td>104</td>
<td>2.3</td>
</tr>
<tr>
<td>90% saturated</td>
<td>30</td>
<td>0.85</td>
<td>25.5</td>
<td>13.6</td>
<td>0.063</td>
<td>76</td>
<td>3.9</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitate dialyzed DEAE-cellulose</td>
<td>22</td>
<td>0.48</td>
<td>10.6</td>
<td>1.96</td>
<td>0.245</td>
<td>32</td>
<td>15.3</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>14</td>
<td>0.53</td>
<td>7.42</td>
<td>0.72</td>
<td>0.736</td>
<td>22</td>
<td>46.0</td>
</tr>
</tbody>
</table>

*μmol Ade released/min·mg protein at 30 C.
around 4.7 with either Ado or i6Ado 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 i6Ado or Ado deribosylation by adenosine nucleosidase reached a maximum in 30 min and then leveled off (Fig. 4, only the data of i6Ado 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 i6Ado deribosylation with respect to enzyme concentration is shown in the inset of Figure 4.

**Effects of Metal Ions and Sulphydryl Reagents.** The effects of the chloride form of K+, Mg2+, Ca2+, and Mn2+ 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 i6Ado was detected in the presence of these metal ions. Various concentrations (0.1-10 mM) of sulphydryl 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) (1:3, v/v) solvent system. The solvent system gave a clear separation of Ado or i6Ado from Ade or i6Ade. Approximate RF values for these compounds were: Ade, 0.36; Ado, 0.64; i6Ade, 0.40 and i6Ado, 0.73. Alternatively, larger quantities of deribosylated nucleoside products were obtained by scaling up of experiments by using 14C-labeled compounds and replacing 14C-labeled substrates with unlabeled ones. The UV absorption spectra of the purified unlabeled deribosylated products were: for i6Ade: max at pH 2, 273 nm; at pH 7, 269 nm; at pH 12, 275 nm; and for Ado: 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.

**DISCUSSION**

The results of these studies demonstrate that i6Ado serves as a substrate for adenosine nucleosidase from wheat germ. Deribosylated nucleosides were obtained from the hydrolysis of synthetic cytokinin ribosides, but not from naturally occurring cytokinin ribosides. These results agree with the values for corresponding authentic compounds.

<table>
<thead>
<tr>
<th>Nucleoside Added</th>
<th>Concentration (mM)</th>
<th>Relative Activity (i6 Ade formed) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.5</td>
<td>100</td>
</tr>
<tr>
<td>3′-Deoxyadenosine</td>
<td>0.5</td>
<td>95</td>
</tr>
<tr>
<td>2′-Deoxyadenosine</td>
<td>0.5</td>
<td>96</td>
</tr>
<tr>
<td>Ado</td>
<td>5.0</td>
<td>96</td>
</tr>
<tr>
<td>N6-Furfuryladenosine</td>
<td>0.5</td>
<td>96</td>
</tr>
<tr>
<td>i6Ado</td>
<td>0.5</td>
<td>96</td>
</tr>
<tr>
<td>N6-Benzyladenosine</td>
<td>0.5</td>
<td>96</td>
</tr>
<tr>
<td>Guanosine</td>
<td>5.0</td>
<td>96</td>
</tr>
<tr>
<td>Uridine</td>
<td>5.0</td>
<td>96</td>
</tr>
</tbody>
</table>

**TABLE III. Kinetic Constants for Adenosine Nucleosidase**

Experimental conditions are as described in Table II, except that the data of K<sub>m</sub> and V<sub>max</sub> were obtained from eight different substrate concentrations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (µM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (µmol/min·mg protein)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ado</td>
<td>1.43</td>
<td>0.71</td>
<td>0.50</td>
</tr>
<tr>
<td>i6Ado</td>
<td>2.38</td>
<td>0.27</td>
<td>0.11</td>
</tr>
</tbody>
</table>
slation of \( i^6 \text{Ado} \) and \( \text{Ado} \) 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 \( \text{Ado} \) by an isopentenyl amino side chain decreases the \( K_m \) value of the reaction by a factor of about 1.7, and \( \text{Ado} \) is about 4.5-fold more efficient than \( i^6 \text{Ado} \) as a substrate (Table III). The adenosine nucleosidase exhibited a specificity for \( \text{Ado} \) and \( N^6 \)-derivatives of \( \text{Ado} \), but not for guanosine, \( 3' \)-deoxyadenosine, or uridine. Furthermore, the naturally occurring cytokinin, \( i^6 \text{Ado} \), was more effective in inhibiting the deribosylation of \( i^6 \text{Ado} \) 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


