Guanosine Deaminase and Guanine Deaminase from Tea Leaves

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Guanosine deaminase and guanine deaminase were partially purified from tea leaves. The optimum activity of guanosine deaminase was observed at pH 7.5 and that of guanine deaminase was at pH 7.0–7.5 and 8.5. Guanosine deaminase was an unstable enzyme. The activities of these deaminases were significantly inhibited by heavy metals. Molecular weights of guanosine deaminase and guanine deaminase as measured by gel filtration were about 18,000 and 54,000, respectively. The Kₘ for the respective substrates, guanosine and guanine, were 9.5 μM and 41.7 μM. Guanosine deaminase was considered to catalyze the deamination of 2'-deoxyguanosine besides guanosine. It is suggested that guanosine deaminase as well as guanine deaminase in tea leaves not only acts on the catabolic pathway, but also is involved in the biosynthesis of caffeine from guanosine or guanine nucleotides.

In the conversion of guanosine to xanthine in vivo, the following two routes are suggested: (i) guanosine → guanine → xanthine and (ii) guanosine → xanthosine → xanthine. Xanthine is further degraded to CO₂ and NH₃ via uric acid. These pathways include two deamination reactions of guanine derivatives, which are catalyzed by guanosine deaminase and guanine deaminase. Most studies on the two deaminases have been done in animal tissues and microorganisms. Guanine deaminases from rat liver and mouse liver, rabbit liver, rat brain, mouse brain, and Clostridium acidurici have been partially purified and characterized. The activity of guanosine deaminase was also detected in rat liver and rat brain. However, the production of xanthine from guanosine in these tissues is considered to be caused by a combination of nucleoside phosphorylase or nucleosidase and guanine deaminase. Guanosine deaminase was first purified and characterized from the cells of Pseudomonas convexus No. 149. The biological roles of the two deaminases in plants are of interest. The activity of guanine deaminase alone was found in tobacco leaf extracts. In a previous study, we detected the activity of guanosine deaminase in cell-free extracts of tea leaves. In these leaves GMP may be converted to xanthosine via guanosine. Our further investigation indicated that guanine deaminase is also present in tea leaves. This paper deals with the partial purification and characterization of guanosine deaminase and guanine deaminase from tea leaves. It seems to be the first report on the purification of these enzymes from plants. We also discuss their roles in caffeine biosynthesis and purine metabolism.

Materials and Methods

Plants. Fresh tea leaves (Camellia sinensis (L.) O. Kuntze, cv. Yubukita) were plucked at a tea garden near the University of Tsukuba in May, 1993, and stored at −20°C in a freezer.

Chemicals. [8-14C]GMP (2.07 GBq/mmol), [8-14C]guanosine (2.07 GBq/mmol), [8-14C]guanine (2.07 GBq/mmol), [8-14C]2'-deoxyguanosine (2.07 GBq/mmol), and [2-14C]cytidine (2.04 GBq/mmol) were purchased from Moravek Biochemicals, Inc., while [8-14C]AMP (2.07 GBq/mmol), [8-14C]adenosine (1.85 GBq/mmol), and [8-14C]cytidine (2.30 GBq/mmol) were purchased from Amersham International plc.

Preparation and purification of the crude enzymes. All the procedures were done at 4°C. Tea leaves (100 g) were homogenized in a blender with 75 g of washed Polycarb AT and 500 ml of 0.1 M NaHPO₄ (pH 7.0) containing 0.6% ascorbic acid. The solution was squeezed through cheesecloth and centrifuged for 10 min at 30,000 × g. The supernatant, which was called the cell-free extract, was brought to 80% saturation by addition of solid (NH₄)₂SO₄ and stirred for 30 min. The precipitate was collected by centrifugation for 10 min at 30,000 × g and dissolved in the NaHPO₄-ascorbic acid solution used above. After centrifugation, the supernatant was dialyzed for 6 h against 0.02 M HEPES–KOH buffer (pH 7.5) containing 1 mM 2-mercaptoethanol. This dialyzed solution was called the crude extract. The purification of the crude enzymes was done with a DEAE-cellulose column (DE-52, Whatman, 1.6 × 35 cm) and a Sephadex G-100 column (1.6 × 95 cm) equilibrated with 0.02 M HEPES–KOH buffer (pH 7.5) containing 1 mM 2-mercaptoethanol.

Assay for enzyme. The assays for deaminases were based on the separation of products from substrates by HPLC or paper chromatography. The reaction mixture for the standard assay for these enzymes contained 0.08 mM HEPES–KOH buffer (pH 7.5) (TAPS–KOH buffer (pH 8.5) was used for guanine deaminase), 44,000 rpm (3.6 × 10⁶ ß–mol) of ¹⁴C-labeled compounds, and enzyme solution in a total volume of 20 μl. The reaction was done at 30°C for 1.5 h and stopped by the addition of 2 μl of 2 N HCl. Then, 4 μl of the carrier solution (1 mM) was added. The mixture (a 5-μl sample) was analyzed with a Shimadzu LC-10A system.
liquid chromatograph. An Inertsil prep-ODS column (GL Science), 4.6 mm i.d. x 250 mm, was used for the separation of nucleosides or bases, and a Partisil 10 SAX column (Whatman), 4 mm i.d. x 250 mm, for the separation of nucleotides. Elution was done with the following solvents: (A) H$_2$O-acetonitrile-CH$_3$COOH-triethylamine (95:4.0:3.0:0.3) for the separation of guanosine and xanthosine, (B) H$_2$O-acetonitrile-CH$_3$COOH-triethylamine (95:2.0:3.0:0.3) for the separation of adenosine and inosine, adenine and hypoxanthine, 2'-deoxyguanosine and 2'-deoxyxanthosine, or cytidine and uridine, (C) 0.02 M KH$_2$PO$_4$ (pH 2.5)-acetonitrile (96:4) for the separation of guanine and xanthine, or cytosine and uracil at the flow rate of 1.1 ml/min, (D) 0.025 M KH$_2$PO$_4$ (pH 3.4) for the separation of AMP and IMP, and (E) 0.05 M KH$_2$PO$_4$-0.1 M KCl (pH 5.0) for the separation of GMP and XMP at the flow rate of 1.5 ml/min. In the measurement of activity for 2'-deoxyguanosine, the reaction mixtures were analyzed by HPLC with solvents (B) and (C) as the reaction product 2'-deoxyxanthosine was hydrolyzed by the acid added to stop the reaction. The eluates were monitored by the absorbance at 260 nm, collected separately, and their radioactivity was measured with a Beckman LS 5000TA liquid scintillation system. For the separation of adenosine and inosine, paper chromatography was also done with a solvent of isobutyric acid-0.5 N ammonia (3:5). One unit was defined as the amount of enzyme which deaminates 1 µmol of substrate per minute under the standard assay conditions.

**Protein measurement.** Protein was measured by the method of Bradford with bovine serum albumin as a standard protein.

**Molecular weight measurement.** The molecular weights of deaminases were estimated by Sephadex G-100 gel filtration. The standard proteins and their molecular weights were: aldolase, 168,000; bovine serum albumin, 68,000; chemotropsigenin A, 25,000; cytochrome c, 12,500.

**Results and Discussion**

**Deamination of guanine derivatives by crude enzyme solutions**

To measure the activities for the deamination, $^{14}$C-labeled guanine derivatives, i.e., GMP, guanosine, and guanine, were each incubated at 30°C for 1.5 h with the cell-free extract prepared from the homogenate of tea leaves or the crude extract obtained by salting-out and dialysis, and the respective deaminated compounds were assayed by HPLC. As shown in Table I, deaminating activities for guanosine and guanine were detected. However, deamination of GMP was unclear owing to the low level of activity for GMP or the degradation of nucleotides by phosphatases in the enzyme solutions. In the cell-free extract, activity for guanosine was about 28 times higher than that for guanine. However, it was only 2.8 times predominant in the crude extract, indicating that guanosine deaminating activity was lost significantly during the salting-out and dialysis procedures.

**Purification of guanosine deaminase and guanine deaminase**

**Step 1. DEAE-cellulose column chromatography.** The crude extract was put on a DEAE-cellulose column and the column was washed with 0.02 M HEPES-KOH buffer (pH 7.5) containing 1 mM 2-mercaptoethanol. Gradient elution was done with NaCl from 0 to 0.5 M in the same buffer. A typical elution pattern of enzyme activities is shown in Fig. 1. Peaks showing guanosine and guanine deaminase activities were eluted at about 0.15 M NaCl, but these activities were not separated from each other.

**Step 2. First Sephadex G-100 gel filtration.** The fractions containing guanosine and guanine deaminase activities obtained from step 1 were pooled, concentrated to 3 ml by ultrafiltration, and put on a Sephadex G-100 column, which was eluted with the same buffer as above. Guanosine deaminase activity and guanine deaminase activity were separated as shown in Fig. 2, indicating the presence of two deaminases.

**Step 3. Second Sephadex G-100 gel filtration.** Each of the deaminase fractions from step 2 was separated again through the same Sephadex G-100 column. One deaminase fraction, which did not contain the other deaminase activity, was obtained. The results of the overall purification are summarized in Table II. Each of the deaminase fractions was pooled and used for the subsequent experiments.

![Fig. 1. Elution Profiles of Guanosine Deaminase and Guanine Deaminase from DEAE-Cellulose.](image)

**Table I. Deamination of Guanine Derivatives by Crude Enzyme Solutions**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity in pmol/min</th>
</tr>
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<tr>
<td></td>
<td>Cell-free extract</td>
</tr>
<tr>
<td>GMP</td>
<td>—</td>
</tr>
<tr>
<td>Guanosine</td>
<td>30,420</td>
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<tr>
<td>Guanine</td>
<td>1,080</td>
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</table>

* Could not be measured.

![Fig. 2. First Gel Filtration of Guanosine Deaminase and Guanine Deaminase on Sephadex G-100.](image)
Table II. Purification of Guanosine Deaminase and Guanine Deaminase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (μU)</th>
<th>Specific activity (μU/mg)</th>
<th>Recovery (%)</th>
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</thead>
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<tr>
<td>Cell-free extract</td>
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<td></td>
</tr>
<tr>
<td>(a)*</td>
<td>925.0</td>
<td>30,400</td>
<td>32.9</td>
<td>100</td>
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<tr>
<td>(b)**</td>
<td>925.0</td>
<td>1,080</td>
<td>1.2</td>
<td>100</td>
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<tr>
<td>Crude extract</td>
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<tr>
<td>(a)</td>
<td>715.0</td>
<td>1,810</td>
<td>2.5</td>
<td>5.9</td>
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<tr>
<td>(b)</td>
<td>715.0</td>
<td>646</td>
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<td>DEAE-Cellulose</td>
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<td>(a)</td>
<td>177.0</td>
<td>1,270</td>
<td>7.2</td>
<td>4.2</td>
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<tr>
<td>(b)</td>
<td>177.0</td>
<td>457</td>
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<td>42.6</td>
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<td>1st Sephadex G-100</td>
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<td>(a)</td>
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<td>(b)</td>
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<td>2nd Sephadex G-100</td>
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<tr>
<td>(a)</td>
<td>9.4</td>
<td>1,340</td>
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<td>(b)</td>
<td>12.5</td>
<td>610</td>
<td>48.8</td>
<td>56.7</td>
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</table>

* Guanosine deaminase.
** Guanine deaminase.

Fig. 3. Effects of pH on Guanosine Deaminase (a) and Guanine Deaminase (b) Activity.

The activities were measured under the standard assay conditions except that the following 0.5M buffers were used: Na-acetate buffer (□), MES-KOH buffer (■), HEPES-KOH buffer (●), TAPS-KOH buffer (○), CHES-KOH buffer (∆-), and CAPS-KOH buffer (▲). Properties of guanosine deaminase and guanine deaminase

Molecular weight. The molecular weights of guanosine deaminase and guanine deaminase, estimated by Sephadex G-100 gel filtration, were about 18,000 and 54,000, respectively, by comparing with the elution volumes of standard proteins. The molecular weight of guanosine deaminase from tea leaves is smaller than that (1 x 10^5 to 2 x 10^5) from Pseudomonas convexa No. 149.8

Effects of pH. The optimum pH for guanosine deaminase was pH 7.5; it was different from that of guanine deaminase, which is about pH 8.5 (Fig. 3). However, guanine deaminase from tea leaves seemed to have double peaks of optimum activity, at pH 7.0-7.5 and pH 8.5, as was the case for guanine deaminase from the rat brain. After keeping the enzymes at various pHs (4 to 11) for 1 h at 30°C, the remaining activities were assayed. Guanosine deaminase was stable at pH 6 to 9, and guanine deaminase, at pH 4 to 9.

Effects of temperature. The effects of temperature on the enzyme activity and stability were examined. Maximum activity was observed at about 40°C in both enzymes and the activities rapidly decreased above 50°C (Fig. 4). The enzyme solutions were stored at 4°C and -20°C for various times. Guanosine deaminase lost about 80% of the activity over a period of 72 h at 4°C, but storage at -20°C enabled us to preserve more than 70% of the activity over 7 days. Guanine deaminase was stable at both temperatures for 2 weeks with only a few percent loss.

Effects of metal ions and reagents. Among the various substances tested, the heavy metal ions Cu^{2+}, Cd^{2+}, Zn^{2+}, and Pb^{2+}, and PCMB, inhibited guanosine deaminase strongly as shown in Table III. Guanine deaminase was strongly inhibited by Zn^{2+}, but the inhibition of guanine deaminase by the other metal ions and reagents was smaller than that of guanosine deaminase.

Substrate specificity. Enzyme activities were measured using 9 purine and pyrimidine compounds as substrates to identify substrate specificities of the enzymes. For fear that the partially purified enzyme solutions contained different deaminases, activities were measured by using enzyme preparations at the two Sephadex G-100 gel filtration steps.
and the relative values to that for guanosine or guanine were compared between the two preparations. The results are shown in Table IV. The guanosine deaminase fraction contained activities for 2'-deoxyguanosine, adenosine, adenine, and cytidine, but no activity toward guanine. The relative activity for 2'-deoxyguanosine hardly changed among the two purification steps, suggesting that activities for both guanosine and 2'-deoxyguanosine were catalyzed by one enzyme. In contrast, the relative activities for adenosine and adenine increased, indicating that adenosine and adenine were deaminated by different enzymes from guanosine deaminase. The relative activity for cytidine, which seems to be due to the contamination of the guanine deaminase fraction, significantly decreased. Guanosine deaminase, which can catalyze 2'-deoxyguanosine, has also been found in *Pseudomonas convexa* No. 149. On the other hand, the guanine deaminase fraction showed no activities for guanosine and 2'-deoxyguanosine, but contained enzyme activities toward adenine, cytidine, and cytosine. This result may be taken to indicate the presence of several deaminases besides guanine deaminase, although the possibility that these activities are caused by a single enzyme cannot be excluded. Purine nucleotide deaminating activities could not be measured as the nucleotides were hydrolyzed by phosphatascontaminating the enzyme fractions. However, it seems that AMP deaminase was not contained in the partially purified enzyme solutions as the molecular weight of AMP deaminase was very large and guanosine deaminase and guanine deaminase can be completely separated from this enzyme by Sephadex G-100 column chromatography (unpublished data).

K<sub>m</sub> values of guanosine deaminase and guanine deaminase. The rates of deamination at various concentrations of guanosine or guanine were calculated for the Lineweaver–Burk plots. The K<sub>m</sub> values for guanosine and guanine were 9.8 μM and 41.7 μM, respectively.

**Roles of guanosine deaminase and guanine deaminase in purine metabolism and caffeine biosynthesis**

The activity of guanosine deaminase contained in tea leaves was about 28 times higher than that of guanine deaminase. The K<sub>m</sub> of guanosine deaminase for guanosine was smaller than that of guanine deaminase for guanine. These facts indicate that the reaction from guanosine to xanthosine, which further converts to xanthine and 7-methylxanthosine, proceeds faster than the reaction from guanine to xanthine in tea leaves.

Caffeine is synthesized from xanthosine via 7-methylxanthosine, 7-methylxanthine, and theobromine. We have indicated that xanthosine was derived from purine nucleotides; the feeding experiment of [14C]guanosine to tea shoots showed that part of the guanosine was directly converted to xanthosine, although most of the guanosine was immediately incorporated into guanine nucleotides. These guanine nucleotides may be converted to guanosine again. Therefore, as shown in Fig. 5, we suggest that guanosine deaminase may function to form xanthosine leading to the pathway of caffeine biosynthesis, although xanthosine is also degraded to xanthine. Xanthine can alternatively be formed from guanosine by a combination of nucleoside phosphorylase or nucleosidase and guanine deaminase in tea leaves, as was reported in microorganisms and animals. Guanine deaminase is involved in the conversion of guanine to xanthine, which is further degraded to CO₂ and NH₃. Therefore, guanine deaminase may mainly act on the pathway of purine catabolism. It is unknown whether guanosine deaminase and guanine deaminase also exist in other plants. The roles of these deaminases in caffeine biosynthesis and purine metabolism will be made clearer by further investigations.

**References**