Purification and Properties of Adenosine Nucleosidases from Tea Leaves

Hiroshi IMAGAWA, Haruko YAMANO,* Kazuhiko INOUE
and Yoshinori TAKINO**

*Institute of Applied Biochemistry, the University of Tsukuba,
Ibaraki-ken 305*

***Department of Agricultural Chemistry, Faculty of Agriculture,
Tamagawa University, Machida-shi, Tokyo 194*

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Three adenosine nucleosidases (adenosine ribohydrolase, EC 3.2.2.7) with high substrate specificity were isolated from the extracts of tea leaves by a procedure including fractionation with ammonium sulfate, column chromatography on DEAE- and CM-cellulose, and gel filtration on Sephadex G-100. They were designated adenosine nucleosidase I, II and III, respectively, and their properties were characterized.

Among the naturally occurring nucleosides only adenosine and 2'-deoxyadenosine were hydrolyzed by these three enzymes and cleavage rate of the N-glycosidic bond in 2'-deoxyadenosine was three or four times greater than that in adenosine.

In the previous paper,¹⁾ the authors reported the degradation of RNA by tea leaf nucleases. Guanosine, uridine and adenine were found in the hydrolysate of RNA by the crude enzyme preparation from tea leaves. These results suggest the presence of the enzyme in tea leaves which will only hydrolyze adenosine among the naturally occurring ribonucleosides.

Adenosine nucleosidase activity was demonstrated in leaves of wheat,²⁾ cabbage,³⁾ Brussels sprouts,⁴⁾ potato^{5,6)} and barley.⁷⁾ The enzymes of potato and barley leaves had been purified to a nearly homogeneous state. Although their substrate specificity and other properties were examined, the metabolic role of these enzymes in plants has not hitherto been defined.

On the other hand, the biosynthesis of caffeine in tea plant has been investigated by several workers. Suzuki *et al.*⁸⁾ suggested that caffeine was biosynthesized from purine nucleotide *via* 7-methylxanthine and theobromine, but little is known about the pathways leading to the formation of 7-methylxanthine. The find-

ing of highly specific nucleosidases in tea leaves will serve to elucidate the problem.

This paper describes the partial purification and some properties of the enzyme.

MATERIALS AND METHODS

Tea leaves. The first two leaves and a bud from fresh tea shoots (*Camellia sinensis* (L.) O. Kuntze, clone Benihomare) were plucked at the field of the National Research Institute of Tea in May, and stored in the freezer.

Chemicals. All ribonucleosides and deoxyribonucleosides were purchased from Kyowa Hakko Kogyo Co., except 6-methyladenosine, 3'-deoxyadenosine and adenosine N₁-oxide, which were obtained from Sigma Chemical Co. Protein molecular weight markers were obtained from Boehringer Mannheim Yamanouchi Co. DEAE- and CM-cellulose were the products of Serva Co., and Sephadex G-100, a product of Pharmacia Co.

Enzyme assay. Adenosine nucleosidase activity was assayed by measuring the amount of ribose or deoxyribose released according to the procedure of Somogyi.⁹⁾ The usual reaction mixture of total volume 1.8 ml contained 0.5 ml of 0.1 M acetate buffer (pH 4.5), 0.5 ml of 12 mM adenosine and 0.8 ml of enzyme solution. After 1 hr of incubation at 25°C, 0.2 ml of 1 N NaOH was added and ribose released was determined. One unit (U) of the activity is defined as the amount of

* Present address: Department of Pathological Biochemistry, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101.

enzyme which catalyzes the hydrolysis of 1 μ mol of adenosine per minute. Phosphomonoesterase (PMase) activity was assayed as previously described,¹⁾ but this time one unit of the activity is defined as the amount of enzyme which produces 1 μ mol of *p*-nitrophenol per minute at 25°C.

Protein was estimated by Lowry's procedure¹⁰⁾ using bovine serum albumin as the standard protein. In column eluates only the absorbance at 280 nm was monitored.

Chromatographic identification of reaction products. Detection and identification of reaction products were carried out by paper chromatography on Whatman No. 1 filter paper using the following solvent systems: (1) water (pH 10), (2) *n*-butanol-isobutyric acid-25% aq. ammonia-water (75:37.5:2.5:25), (3) *n*-butanol-acetic acid-water (4:1:5). Upon viewing the paper in UV light, spots of nucleosides and their respective bases were detected. Ribose was detected as a reddish brown spot by spraying with an aniline hydrogen phthalate followed by incubation for 5 min at 105°C.

Molecular weight determination. The molecular weight of adenosine nucleosidase was determined by Sephadex gel filtration technique. The column of Sephadex G-100 (1.5 $\times$ 90 cm) equilibrated with 0.01 M acetate buffer (pH 5.6) was calibrated with following marker proteins: cytochrome C (12,500), chymotrypsinogen A (25,000), ovalbumin (45,000), bovine serum albumin (68,000) and aldolase (158,000).

RESULTS AND DISCUSSION

Purification of the enzyme

The procedure is summarized in Table I. All operations were carried out in the cold at 4°C.

Step 1. Crude extract. Three hundred and sixty grams of tea leaves was homogenized in cold acetone for 1 min. The homogenate was filtered through filter paper with suction and the residue was washed three times with 4:1 acetone to remove polyphenols. Acetone powder thus obtained was extracted for 2 hr with 0.1 M phosphate buffer (pH 7.0), containing 1% of sodium ascorbate.

Step 2. Ammonium sulfate fractionation. The crude extract was centrifuged at 28,000 $\times g$ for 20 min. Solid ammonium sulfate was added slowly with stirring to the solution to bring 80% saturation. After standing overnight, the precipitate was collected by centri-

fugation and suspended in 0.01 M phosphate buffer, pH 7.0. The suspension was dialyzed against the same buffer for 24 hr. After dialysis, precipitate was removed and discarded. This solution was refractionated with solid ammonium sulfate. The precipitate obtained between 40% to 80% saturation was dissolved in 0.04 M phosphate buffer (pH 7.0) and dialyzed against the same buffer.

Hydrolysis of ribonucleosides by crude enzyme.

Five μ mol of each substrate was incubated with the crude enzyme (dialyzed enzyme solution, step 2) in acetate buffer (pH 5.0) at 25°C for 4 hr. At intervals of 30 min, an aliquot (10 μ l) of the reaction mixture was applied to a filter paper and developed with solvent (1), (2) and (3). Adenine and ribose were identified as the reaction products, when adenosine was supplied as substrate. No reaction products were observed in the incubation of guanosine, cytidine and uridine.

Further, six kinds of ribonucleosides (adenosine, guanosine, inosine, xanthosine, cytidine and uridine) and four kinds of 2'-deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine) were incubated with the crude enzyme solution (14 mU) at 25°C for 1 hr, and ribose or deoxyribose released was assayed. Only adenosine and 2'-deoxyadenosine were hydrolyzed among them, and relative activity toward the latter compound was about four times higher than that toward the former.

These results show that adenosine nucleoside with high specificity exist in the crude extracts from tea leaves and the other nucleosidases are absent.

Step 3. DEAE-cellulose column chromatography. The dialyzed enzyme solution was applied to a DEAE-cellulose column (1.6 $\times$ 30 cm) equilibrated previously with 0.04 M phosphate buffer, pH 7.0, and the column was washed with 750 ml of the same buffer. The washing contains about 81% of the total PMase activity of the crude enzyme. Elution was continued successively with 0.1 M phosphate buffer (pH 7.0) and a linear gradient of

FIG. 1. Chromatography of the Extract from Tea Leaf on DEAE-Cellulose Column.

Column size, 1.6×30 cm; flow rate, 25 ml/hr; fraction volume, 5 ml. $\circ$ — $\circ$, adenosine nucleosidase activity; $\bullet$ — $\bullet$, PMase activity.

FIG. 2. Chromatography of Adenosine Nucleosidase Fraction (step 3) on Sephadex G-100 Column.

Column size, 1.5×90 cm; flow rate, 6.0 ml/hr; fraction volume, 2 ml. $\circ$ — $\circ$, adenosine nucleosidase activity; $\bullet$ — $\bullet$, PMase activity; ----, E_{280} .

zero to 0.5 M NaCl in the same buffer. Adenosine nucleosidase activity was found in fractions between Nos. 194 and 215 (Fig. 1). Active fractions were pooled and concentrated with membrane ultra filter (Amicon PM-10) to about 10 ml.

Step 4. First gel filtration on Sephadex G-100. The enzyme solution from step 3 was applied to a Sephadex G-100 column (1.5×90 cm) equilibrated previously with 0.04 M acetate buffer, pH 5.6. Filtration was carried out with the same buffer. As shown in Fig. 2, adenosine nucleosidase activity was separated from PMase activity. The active fractions

FIG. 3. Chromatography of Adenosine Nucleosidase Fraction (step 4) on CM-cellulose Column.

Column size, 1.6×30 cm; flow rate, 14.6 ml/hr; fraction volume, 4 ml. $\circ$ — $\circ$, adenosine nucleosidase activity; ----, E_{280} .

(Nos. 32 to 44) were collected and concentrated with membrane ultra filter to about 10 ml.

Step 5. CM-cellulose column chromatography. The enzyme solution from step 4 was applied to a CM-cellulose column (1.6×30 cm) equilibrated previously with 0.04 M acetate buffer, pH 5.6. After washing the column with 320 ml of the same buffer, the enzymes were eluted with 0.1 M acetate buffer (pH 5.6) and linear gradient of zero to 0.5 M NaCl in the same buffer in the order. As shown in Fig. 3, three peaks having adenosine nucleosidase activity were obtained. Peak I (Nos. 18 to 24), II (Nos. 98 to 108) and III (Nos. 170 to 200) will be called adenosine nucleosidase I, II and III, respectively. Each of the enzyme fractions was concentrated to 1 ml with amicon ultra filter.

Step 6. Second gel filtration on Sephadex G-100. Each of the concentrated enzyme solution from step 5 was subjected to rechromatography on Sephadex G-100 column by the same method as step 4. Adenosine nucleosidase activity in each enzyme fraction was eluted as a single peak of fraction Nos. 34 to 40 (enzyme I), 34 to 44 (II) and 34 to 42 (III), respectively. These three enzyme preparations were all free of nucleotidases, nonspecific phosphatases and other nucleosidases. The purified enzymes were stable for at least one month

TABLE I. PURIFICATION OF ADENOSINE NUCLEOSIDASES FROM TEA LEAVES

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Ammonium sulfate (80%)	1110	112	0.10	100
Ammonium sulfate (40~80%)	235	94.1	0.40	84.8
DEAE-cellulose	81.2	79.1	0.97	71.3
1st Sephadex G-100	12.9	45.1	3.50	40.6
CM-cellulose				
Peak I	1.84	6.90	3.74	6.2
II	1.85	17.28	9.35	15.4
III	1.36	8.31	6.11	7.4
2nd Sephadex G-100				
Enzyme I	0.70	4.90	7.00	4.4
II	0.81	10.41	12.84	9.3
III	0.74	5.90	7.97	5.3

when stored at -20°C , but gradually lost their activity on storage at 4°C .

The results of the overall purification are summarized in Table I.

Properties of adenosine nucleosidases

Molecular weight. The elution volume of the three adenosine nucleosidases from gel filtration of Sephadex G-100 as described in the "Methods", indicated that the molecular weight of enzymes I, II and III were all approximately 68,000. This value is similar to

FIG. 4. pH-Activity Curves of Adenosine Nucleosidases.

The amounts of enzyme I, II and III used were 5.5, 4.6 and 3.1 mU, respectively. The following buffers were used: sodium acetate-HCl (pH 2.0 to 4.5), sodium acetate-acetic acid (pH 3.5 to 6.0), sodium phosphate (pH 6.0 to 8.0). ○—○, enzyme I; ●—●, enzyme II; △---△, enzyme III.

that of adenosine nucleosidase from barley leaves (66,000).

Effect of pH. The effect of pH on the enzyme activity was investigated over the pH range from 2.0 to 8.0. The highest activity was observed at about pH 4.0 for enzymes I and III, and at pH 4.5 for enzyme II (Fig. 4).

pH stability. Each enzyme solution in 0.02 M acetate buffer (pH 3.5 to 5.8) and 0.02 M phosphate buffer (pH 6.0 to 8.0) was kept at 4°C for 18 hr and respective activity was assayed. The three adenosine nucleosidases were all unstable in acidic media, but stable in the range of pH 6.0 to 8.0 (Fig. 5).

Effect of temperature. Effect of tempera-

FIG. 5. pH-Stability of Adenosine Nucleosidases.

Each solution of enzyme I (11.0 mU), II (9.2 mU) and III (9.0 mU) was kept at 4°C for 15 hr at various pH (pH 3.5 to 5.0, acetate buffer; pH 6.0 to 8.0, phosphate buffer). After adjustment of pH to 4.5, the remaining activity was assayed. ○—○, enzyme I; ●—●, enzyme II; △---△, enzyme III.

FIG. 6. Temperature-Activity Curves of Adenosine Nucleosidases.

Each solution of enzyme I (11.0 mU), II (6.0 mU) and III (6.0 mU) was added to the adenosine solution and incubated for 30 min at various temperatures. ○—○, enzyme I; ●—●, enzyme II; △---△, enzyme III.

ture on the enzyme activity was examined over a range from 25° to 60°C. As shown in Fig. 6, the maximum activity occurred at 45°C for enzyme I and at 50°C for both enzymes II and III.

Thermal stability. Each of adenosine nucleosidases I, II and III in 0.02 M phosphate buffer, pH 7.0, was kept at various temperatures for 15 min. After cooling, the residual activity was assayed under the standard con-

FIG. 7. Thermal Stability of Adenosine Nucleosidases. Each solution (0.3 ml) of enzyme I (10.4 mU), II (11.6 mU) and III (12.0 mU) in 0.02 M phosphate buffer (pH 7.0) was kept various temperatures for 15 min, and remaining activity was assayed. ○—○, enzyme I; ●—●, enzyme II; △—△, enzyme III.

TABLE II. EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF ADENOSINE NUCLEOSIDASES

After each enzyme preparation in 1.0 ml of 0.05 M acetate buffer pH 4.5 (I, 10.0 mU; II and III 8.5 mU) was preincubated at 25°C for 10 min in the presence of various substances, enzyme activity was measured under the standard assay conditions.

Substance	Final conc. (mM)	Relative activity (%)		
		Enzyme I	Enzyme II	Enzyme III
None		100	100	100
NaF	1	93	81	104
MgCl ₂	1	102	89	109
CaCl ₂	1	102	87	97
FeSO ₄	1	109	79	84
CoCl ₂	1	99	77	72
CuSO ₄	1	97	79	90
ZnSO ₄	1	99	78	109
EDTA	1	106	89	95
ICH ₂ COOH	1	101	100	99
PCMB	0.1	86	104	96
Adenine	0.2	102	75	—
	0.5	101	70	101
	1	97	60	98
	4	56	37	70

ditions. Enzymes I, II and III rapidly lost their activity above 40°C (Fig. 7).

Effect of metal ions and inhibitors. Table II indicates that metal ions and reagents tested did not give any serious effects to the enzyme activity at 1 mM concentration. One of the products, adenine, showed different inhibitory effect on the enzymes. Adenosine nucleosidase II was inhibited at low concentration of adenine (0.2 mM), while both adenosine nucleosidases I and III were not inhibited up to 1.0 mM.

Substrate specificity. There was a remarkable similarity in the substrate specificity of the three adenosine nucleosidases. They hydrolyzed only adenosine and 2'-deoxyadenosine among the naturally occurring nucleosides, and cleavage rate of *N*-glycosidic bond in 2'-deoxyadenosine was three or four times greater than that in adenosine. Of the other nucleosides tested only adenosine N₁-oxide was readily hydrolyzed (Table III). It seems necessary for the hydrolysis of the nucleoside by these enzymes that the hydrogens of 6-amino group

TABLE III. SUBSTRATE SPECIFICITY OF ADENOSINE NUCLEOSIDASES

The reaction mixture containing each substrate (6 μmol), 0.1 M acetate buffer (pH 4.0 for enzyme I and III, pH 4.5 for enzyme II) and enzyme* was incubated at 25°C for 1 hr.

Substrate	Relative activity (%)		
	Enzyme I	Enzyme II	Enzyme III
Adenosine	100	100	100
Guanosine	0	0	0
Inosine	0	0	0
Xanthosine	0	0	0
Cytidine	0	0	0
Uridine	0	0	0
6-Methyladenosine	0	0	0
2'-Deoxyadenosine	421	343	445
2'-Deoxyguanosine	0	0	0
2'-Deoxycytidine	0	0	0
Thymidine	0	0	0
3'-Deoxyadenosine	0	0	0
3'-Adenylic acid	0	0	0
Adenosine N ₁ -oxide	198	172	54

* The amounts of enzymes used were 4.5 mU for 2'-deoxyadenosine and 9.0 mU for the other substrates.

attached to the purine ring is not substituted, and the hydroxyl group at 3'-position of the sugar part is also indispensable. However, the absence of hydroxyl group at 2'-position rather increased the rate of hydrolysis.

High specific activity for both adenosine and 2'-deoxyadenosine was also recognized in the enzymes from other sources.^{6,7)} However, distinct difference was observed in the cleavage rate of the N-glycosidic bond of 2'-deoxyadenosine.^{6,7)} While the enzymes from the leaves of potato⁶⁾ and barley⁷⁾ hydrolyzed 2'-deoxyadenosine at a similar rate to the hydrolysis of adenosine, the enzymes in this paper hydrolyzed 2'-deoxyadenosine much faster than adenosine.

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