N-Methyl Nucleosidase from Tea Leaves

Osamu NEGISHI,* Tetsuo OZAWA and Hiroshi IMAGAWA**

Institute of Applied Biochemistry, University of Tsukuba,
Sakura-mura, Niihari-gun, Ibaraki 305, Japan

Received August 10, 1987

N-Methyl nucleoside hydrolase (N-methyl nucleosidase, N-MeNase), which hydrolyzes 7-methylxanthosine to produce 7-methylxanthine, was detected in tea-leaf extracts and separated from adenosine nucleosidase (ANase, EC 3.2.2.7) by DEAE-cellulose column chromatography.

The optimum pH for the N-MeNase ranged from 8.0 to 8.5. The enzyme was strongly inhibited by EDTA. Inhibition by the hydrolysis products of 7-methylxanthosine and 7-methylinosine was also observed. The molecular weight was estimated to be about 55,000 by gel-filtration.

Among purine and N-methylpurine nucleosides, 3- and 7-methylpurine nucleosides were hydrolyzed preferentially by N-MeNase. On the other hand, ANase could not hydrolyze 7-methylxanthosine, although the enzyme showed high activity toward 7-methyladenosine. As a result, it is suggested that N-MeNase catalyzes the hydrolysis reaction of 7-methylxanthosine in the pathway of caffeine biosynthesis, whereas ANase is not directly concerned with it.

In the previous papers we suggested the presence of a nucleosidase or nucleoside phosphorylase, which degrades 7-methylxanthosine to produce 7-methylxanthine, in cell-free extracts of tea¹⁾ and coffee²⁾ leaves (Fig. 1). The presence of a nucleosidase was also suggested in cell-free extracts of coffee fruits^{3,4)} and coffee callus cultures.^{4,5)} However, detailed studies on the enzyme have not been performed hitherto.

Several nucleosidases have been isolated from various plants, e.g., inosine nucleosidase (EC 3.2.2.2) from yellow lupin seeds⁶⁾ and

adenosine nucleosidase (EC 3.2.2.7) from barley leaves,⁷⁾ whereas reports on nucleoside phosphorylase in plants are limited.⁸⁾

Adenosine nucleosidase (ANase) was isolated from tea leaves and characterized.⁹⁾ However, it remains to be determined whether this nucleosidase hydrolyzes 7-methylxanthosine. Recently, we observed that the degradation of 7-methylxanthosine is caused by another enzyme.

This paper deals with the partial purification and characterization of the enzyme degrading 7-methylxanthosine, N-methyl nucleosidase

FIG. 1. Biosynthesis of Caffeine from 7-Methylxanthosine in Tea and Coffee Plants.

(A) nucleosidase; (B) nucleoside phosphorylase; R-1-P, ribose-1-phosphate; SAM, S-adenosylmethionine.

* Present address: Tokyo Research Laboratory, Takasago International Corporation, 5-36-31 Kamata, Ōta-ku, Tokyo 144, Japan.

** To whom correspondence should be addressed.

Abbreviations: PCMB, *p*-chloromercuribenzoate; Tricine, Tris(hydroxymethyl)methylglycine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

(N-MeNase). We also discuss the role of N-MeNase and ANase in the biosynthesis of caffeine and purine metabolism.

MATERIALS AND METHODS

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

Chemicals. 7-Methylxanthosine, 7-methylinosine, 7-methylguanosine, 1-methyladenosine, 1-methylinosine, 1-methylguanosine, 1-methyladenine and 3-methylxanthine were purchased from Sigma Chemical Co. 7-Methyladenosine, 3-methyladenosine, 3-methylguanosine, 3-methylinosine and 3-methylxanthosine were kindly provided by Professor T. Fujii, Faculty of Pharmaceutical Sciences, Kanazawa University. 1-Methylxanthosine was generously provided by Dr. A. Yamazaki, Central Research Laboratories, Ajinomoto Company Inc. The 1-, 3- and 7-methylpurine bases except those described above were prepared in our laboratory through hydrolysis of the corresponding nucleosides.

Assay for enzyme activity. The assay for N-MeNase is based on the separation of 7-methylxanthine from 7-

methylxanthosine by high performance liquid chromatography (HPLC).

The standard assay mixture contained $50\text{ }\mu\text{l}$ each of 0.1 M Tricine-NaOH buffer (pH 8.5), 7-methylxanthosine (3.35 mM) and enzyme solution. The reaction was carried out at 37°C for 1 hr and terminated by the addition of $20\text{ }\mu\text{l}$ of 0.5 M HClO_4 . As the internal standard, $30\text{ }\mu\text{l}$ of theobromine (1.34 mM) was added to the reaction mixture. The reaction product was analyzed with a HPLC (TRI ROTAR; Japan Spectroscopic Co., Ltd.). After centrifugation of the reaction mixture at $1,600 \times g$ for 5 min, $2\text{ }\mu\text{l}$ of the supernatant was injected into the ODS column, $4\text{ mm i.d.} \times 300\text{ mm}$ in size (LS-410; Toyo Soda Manufacturing Co., Ltd.) and elution was carried out with 5% AcOH-MeOH ($80:20$) at the flow rate of 1 ml/min . The eluate was monitored as the absorbance at 270 nm with a UV spectrophotometer (UVIDEC-100-III; Japan Spectroscopic Co., Ltd.). Under these conditions, 7-methylxanthosine (t_R : 2.7 min), 7-methylxanthine (t_R : 4.5 min) and theobromine (t_R : 7.3 min) were completely separated from each other. Peak areas were calculated by the internal standard method with an integrator (Model 5000E; System Instruments Co., Ltd.).

ANase activity of the eluates from the DEAE-cellulose and Sephadex G-100 columns was assayed by the Somogyi-Nelson method, as described previously.⁹⁾

One unit was defined as the amount of enzyme which hydrolyzes $1\text{ }\mu\text{mol}$ of substrate per 1 minute under the

FIG. 2. Retention Times of Purine Nucleosides and Bases on HPLC Analysis.

Column, UNISIL PACK 5C18-250A ($4.6\text{ mm i.d.} \times 250\text{ mm}$); solvent, $\text{H}_2\text{O-acetonitrile-AcOH-triethylamine}$ ($95:3:0.3:0.3$); flow rate, 1 ml/min ; temperature, 22°C .

Detection: A_{250} for Guo, Gua, 1MeGuo, 1MeGua, Ino, Hyp, 1MeIno, 1MeHyp, 7MeIno, 7MeHyp; A_{260} for Ado, Ade, 1MeAdo, 1MeAde, 3MeIno, 3MeHyp; A_{270} for 3MeAdo, 3MeAde, 7MeAdo, 7MeAde, 3MeGuo, 3MeGua, 7MeGuo, 7MeGua, Xao, Xan, 1MeXao, 1MeXan, 3MeXao, 3MeXan, 7MeXao, 7MeXan.

Abbreviations for nucleosides and bases: Ado, adenosine; Ino, inosine; Guo, guanosine; Xao, xanthosine; Ade, adenine; Hyp, hypoxanthine; Gua, guanine; Xan, xanthine.

standard assay conditions.

In the experiment on substrate specificity, the assaying of both enzymes was carried out by the HPLC method. The assay conditions were changed as follows because *N*-methylated nucleosides are unstable under acidic and alkaline conditions.^{10,11} The buffers used were 0.1 M HEPES-NaOH (pH 7.5) for N-MeNase and 0.1 M sodium acetate (pH 5.5) for ANase. The reaction mixture comprised 40 μ l of the buffer, 40 μ l of 3.75 mM substrate and 20 μ l of enzyme solution. The reaction was performed at 37°C for 1 hr and stopped by freezing the reaction mixture immediately in a cold MeOH bath (-50°C). The column and solvent used for HPLC were an ODS column, 4.6 mm i.d. $\times$ 250 mm in size (UNISIL PACK 5C18-250 A; Gasukuro Kogyo Inc.) and H₂O-acetonitrile-AcOH-triethylamine (95:3:0.3:0.3), respectively. The nucleosides and the corresponding bases were detected at the wavelengths indicated in the legend to Fig. 2. They were all separated from each other (Fig. 2), and the bases were quantitatively determined.

Identification of the degradation products of 7-methylxanthine. One of the reaction products, 7-methylxanthine, was identified by HPLC as described above. The other one, derived from the sugar moiety, was characterized by paper chromatography. The standard reaction mixture incubated at 37°C for 4 hr was chromatographed using Whatman No. 1 filter paper in *n*-BuOH-AcOH-H₂O (4:1:5), and then reducing sugar was detected by spraying with aniline hydrogen phthalate followed by heating for 5 min at 105°C.

RESULTS AND DISCUSSION

Preparation and properties of the crude enzyme

All the enzyme purification procedures were performed at 4°C.

Tea leaves (1 kg) were homogenized in several separate batches with cold acetone for 3 min. The homogenate was filtered through a filter paper with suction and the residue was washed three times with a mixture of acetone and water (4:1) containing 0.1% ascorbic acid to remove the polyphenols. The acetone powder thus obtained was extracted for 1 hr with 0.1 M disodium hydrogen phosphate solution (pH 7.0) containing 0.6% ascorbic acid. The solution was squeezed through cheesecloth and then centrifuged for 30 min at 3,300 $\times$ *g*. Solid ammonium sulfate was added to the crude extract to 70% saturation, followed by stirring for 30 min. The precipitate was collected by centrifugation for 20 min at

TABLE I. DEGRADATION OF RIBONUCLEOSIDES BY THE CRUDE ENZYME

Substrate	Activity* (nmol/min)
Adenosine	376,000
Guanosine	510
Inosine	113
Xanthosine	2,060
7-Methylxanthosine	6,560

* The assay conditions were the same as in the experiment on substrate specificity. The buffers used were 0.1 M Tricine-NaOH (pH 8.5) for 7-methylxanthosine and 0.1 M sodium acetate (pH 5.0) for the other substrates.

30,000 $\times$ *g* and then dissolved in the extraction solvent used above. After centrifugation, the supernatant was dialyzed for 20 hr against 0.01 M Na-phosphate buffer (pH 7.5).

Five kinds of purine ribonucleosides (adenosine, guanosine, inosine, xanthosine and 7-methylxanthosine) were incubated with the crude enzyme solution at 37°C for 1 hr, and the bases released were assayed by HPLC. As shown in Table I, besides adenosine nucleosidase activity, degrading activity toward 7-methylxanthosine was also detected. The other nucleosides can also serve as substrate, but their cleavage rate were relatively low.

Purification of N-methyl nucleosidase

Step 1. DEAE-Cellulose column chromatography. The dialyzed enzyme solution was applied to a DEAE-cellulose column (2.5 $\times$ 28 cm) equilibrated with 0.01 M Na-phosphate buffer (pH 7.5) and then the column was washed with the same buffer. Elution was performed successively with 0.01 M, 0.05 M and 0.1 M Na-phosphate buffer (pH 7.5). A typical enzyme activity elution pattern is shown in Fig. 3. A peak showing 7-methylxanthosine degrading activity was eluted at 0.05 M Na-phosphate and thus separated from ANase activity, which was eluted at 0.1 M Na-phosphate. A minor peak of another purine nucleosidase was found in the 0.01 M Na-phosphate fraction.

Step 2. Hydroxylapatite column chroma-

FIG. 3. Elution Profiles of *N*-Methyl Nucleosidase and Adenosine Nucleosidase on a DEAE-Cellulose Column.

Column size, 2.5 × 28 cm; flow rate, 32 ml/hr; fraction volume, 16 ml/tube. Enzyme activities: 7MeXao, ●—●; Ado, ○—○; Xao, ◇—◇. Absorbance at 280 nm, -----.

topography. The fractions containing *N*-MeNase activity obtained on DEAE-cellulose column chromatography were applied to a hydroxylapatite column (2.0 × 12 cm) previously equilibrated with 0.01 M Na-Phosphate buffer (pH 7.5). After washing the column with the same buffer, the enzyme was eluted successively with 0.04 M, 0.08 M and 0.16 M Na-phosphate buffer (pH 7.5). *N*-MeNase activity and a small amount of xanthosine degrading activity were eluted in the 0.08 M buffer fraction, and no other peak of nucleosidase activity being detected.

Step 3. Gel filtration on Sephadex G-100. The enzyme solution from step 2 was concentrated to 1 ml by ultrafiltration with an Amicon PM-10 membrane and then chromatographed on a Sephadex G-100 column (2.0 × 82 cm) equilibrated with 0.01 M Na-phosphate buffer (pH 7.5). *N*-MeNase activity was eluted as a single peak and a small amount of contaminating adenosine degrading activity was eluted separately. Xanthosine degrading activity was not detected. The results of the overall purification are summarized in Table

TABLE II. PURIFICATION OF *N*-METHYLNUCLEOSIDASE FROM TEA LEAVES

Step	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield
Ammonium sulfate	1113	6560	5.9	100
DEAE-Cellulose	355	4150	11.7	63
Hydroxylapatite	61.5	1570	25.5	24
Sephadex G-100	12.6	370	29.4	5.6

II.

The *N*-MeNase fractions were pooled and concentrated to 134 mU/ml, and used for the subsequent experiments.

Properties of N-methyl nucleosidase

Molecular weight. The molecular weight was estimated to be about 55,000 by Sephadex G-100 column chromatography, through comparison with the elution volumes of standard proteins (aldolase, 168,000; bovine serum albumin, 68,000; ovalbumin, 45,000; chymotrypsinogen A, 25,000; and cytochrome *c*, 12,000).

Mode of action. The sugar portion of the

reaction products was detected as a pink spot on the chromatogram after spraying with the reagent. Its *R_f* value corresponded with that of an authentic sample of D-ribose. This indicates that the present enzyme cleaves 7-methylxanthosine hydrolytically and not phosphorolytically.

Effect of pH. The optimum pH, from 8.0 to 8.5 (Fig. 4), was considerably different from that for adenosine nucleosidase, *i.e.*, pH 4.5.⁹⁾ After keeping the enzyme at various pHs for 24 hr at 4°C, the remaining activity was as-

FIG. 4. Effect of pH on the Enzyme Activity.

The reaction was carried out under the standard assay conditions. The buffers used were PIPES-NaOH (●), Tricine-NaOH (○) and glycine-NaOH (▲).

FIG. 5. pH-Stability.

The enzyme solution was kept at 4°C for 24 hr at various pHs (▲, sodium acetate; △, PIPES-NaOH; ●, Tricine-NaOH; ○, glycine-NaOH). After adjustment of the pH to 8.5, the remaining activity was assayed.

sayed. The enzyme was unstable in acidic media (Fig. 5).

Effects of temperature. The effects of temperature on the enzyme activity and stability were examined. Maximum activity was observed at temperatures ranging from 40 to 45°C and the activity rapidly decreased above 50°C. The enzyme solution was kept at various temperatures for 15 min. After cooling, the remaining activity was assayed under the standard conditions. The enzyme rapidly lost its activity above 40°C.

Effects of metal ions and other compounds. Among the various substances tested, EDTA was a potent inhibitor (Table III). 7-Methylxanthine and 7-methylhypoxanthine at 5 mM inhibited about 50% of the enzyme activity.

Substrate specificity. Enzyme activity was measured using 16 purine nucleosides, including four free purine nucleosides and their N-methylated derivatives, as substrates. The specificity of ANase (specific activity: 4.0 units/mg), which was partially purified by column chromatography on DEAE-cellulose and Sephadex G-100, was also examined.

TABLE III. EFFECTS OF METAL IONS AND REAGENTS ON THE N-METHYL NUCLEOSIDASE ACTIVITY

Substance (mM)	Relative activity (%)
None	100
MgCl ₂ (1)	105
CaCl ₂ (1)	69
FeSO ₄ (1)	104
CoCl ₂ (1)	101
CuSO ₄ (1)	83
ZnSO ₄ (1)	90
NaF (1)	104
EDTA (1)	11
ICH ₂ COOH (1)	107
PCMB (0.5)	19
7MeXan (1)	95
(5)	60
7MeHyp (1)	103
(5)	50

The results are shown in Table IV. N-McNase hydrolyzed the nucleosides except guanosine and 1-methylguanosine. Among the

TABLE IV. SUBSTRATE SPECIFICITIES OF *N*-METHYL NUCLEOSIDASE AND ADENOSINE NUCLEOSIDASE

The final concentrations of substrates were 1.5 mM. The incubation was carried out at 37°C for 1 hr, with 1.5 mU of *N*-methyl nucleosidase in 0.1 M HEPES buffer (pH 7.5) or 0.5 mU of adenosine nucleosidase in 0.1 M acetate buffer (pH 5.5).

Substrate ^a	Relative activity (%)	
	<i>N</i> -McNase	ANase
Ado	6	100 ^e
1MeAdo	22	1
3MeAdo ^b	178	7
7MeAdo ^c	13	515
Guo	0	0
1MeGuo	0	0
3MeGuo	32	0
7MeGuo	189	0
Ino	5	0
1MeIno	9	0
3MeIno	128	0
7MeIno	121	0
Xao	8	0
1MeXao	12	0
3MeXao	198	0
7MeXao	100 ^d	0

^a See Fig. 2 for abbreviations for substrates.

^b 3-Methyladenosine *p*-toluenesulfonate.

^c 7-Methyladenosine perchlorate.

^d 73.8 nmol of 7-methylxanthine was released into the reaction mixture.

^e 46.9 nmol of adenine was released into the reaction mixture.

substrates tested, the 3- and 7-methylpurine nucleosides were hydrolyzed preferentially by this enzyme. These facts indicate that a methyl group attached at the 3- or 7-position of the nucleosides is important for the enzyme activity. On the other hand, ANase showed high activity toward adenosine and 7-methyladenosine, but the activity toward other substrates was very low or nil. It had already been reported⁹⁾ that ANase hydrolyzes only adenosine among free ribonucleosides. Among the methylated nucleosides tested here, 7-methyladenosine was hydrolyzed five times faster than adenosine, whereas 7-methylxanthosine was not hydrolyzed at all.

Role of nucleosidases in caffeine biosynthesis

In the previous papers^{1,2,12)} we indicated that the pathway for caffeine biosynthesis is as follows: xanthosine→7-methylxanthosine→7-methylxanthine→theobromine→caffeine. This pathway involves the degradation reaction for the *N*-glycosyl bond of methylated nucleoside. Based on the results as to the substrate specificities of the nucleosidases it is evident that the enzyme responsible for the hydrolysis of 7-methylxanthosine is *N*-McNase. Thus ANase does not directly catalyze the reaction in the pathway of caffeine biosynthesis. In plants, ANase is regarded as a member of the adenine salvage pathway.⁷⁾ The same role is considered for ANase from tea leaves. Suzuki and Takahashi¹³⁾ demonstrated that the radioactivity of [8-¹⁴C]adenine is incor-

FIG. 6. Adenine Salvage Pathway and Caffeine Biosynthesis.

a) Adenosine nucleosidase; b) *N*-methyl nucleosidase; c) purine nucleosidase.

porated into RNA purine nucleotides and caffeine. This indicates the presence of the adenine salvage pathway in tea plants. Recently, Suzuki and Waller¹⁴⁾ indicated that caffeine was mainly derived from the nucleotides produced through the purine salvage pathway rather than from those synthesized *de novo*.

Besides the caffeine biosynthetic pathway, there is the catabolic pathway from xanthosine to allantoin and other metabolites.²⁾ In the present study, the presence of an enzyme which hydrolyzes xanthosine was detected in the crude extracts of tea leaves. Characterization of the enzyme responsible for the degradation of xanthosine remains to be performed.

The roles of nucleosidases in tea leaves, related to purine metabolism and caffeine biosynthesis, are illustrated in Fig. 6.

Acknowledgments. The authors wish to express their thanks to Professor Tozo Fujii, Faculty of Pharmaceutical Sciences, Kanazawa University, and Dr. Akihiro Yamazaki, Central Research Laboratories, Ajinomoto Company Inc., for kindly supplying the methylated nucleosides.

REFERENCES

- 1) O. Negishi, T. Ozawa and H. Imagawa, *Agric. Biol. Chem.*, **49**, 887 (1985).
- 2) O. Negishi, T. Ozawa and H. Imagawa, *Agric. Biol. Chem.*, **49**, 2221 (1985).
- 3) M. F. Roberts and G. R. Waller, *Phytochemistry*, **18**, 451 (1979).
- 4) T. Suzuki and G. R. Waller, Abstracts of Papers, 23rd Ann. West Cent. States Biochem. Conf., Columbia, Mo., Paper No. 53, 1980.
- 5) G. R. Waller, C. D. MacVean and T. Suzuki, *Plant Cell Reports*, **2**, 109 (1983).
- 6) A. Guranowski, *Plant Physiol.*, **70**, 344 (1982).
- 7) A. Guranowski and Z. Schneider, *Biochim. Biophys. Acta*, **482**, 145 (1977).
- 8) C-M Chen and B. Petschow, *Plant Physiol.*, **62**, 871 (1978).
- 9) H. Imagawa, H. Yamano, K. Inoue and Y. Takino, *Agric. Biol. Chem.*, **43**, 2337 (1979).
- 10) T. Itaya and H. Matsumoto, *Tetrahedron Lett.*, **1978**, 4047.
- 11) T. Fujii, T. Itaya and T. Saito, *Yuki Gosei Kagaku Kyokaishi*, **41**, 1193 (1983).
- 12) O. Negishi, T. Ozawa and H. Imagawa, *Agric. Biol. Chem.*, **49**, 251 (1985).
- 13) T. Suzuki and E. Takahashi, *Phytochemistry*, **15**, 1235 (1976).
- 14) T. Suzuki and G. R. Waller, *Nippon Dojyo-Hiryogaku Zasshi*, **57**, 70 (1986).