Partial Purification and Properties of Tea Leaf Ribonuclease[†]

Tojiro TSUSHIDA and Tadakazu TAKEO

National Research Institute of Tea, Kanaya-cho, Haibara-gun, Shizuoka-ken, Japan

Received November 11, 1975

Four fractions with ribonuclease activity have been isolated from tea leaves by DEAE-cellulose column chromatography and designated as RNase Tf-1, RNase Tf-2, RNase Tf-3 and RNase Tf-4. The bigger fractions of both RNase Tf-3 and RNase Tf-4 have been partially purified by Sephadex G-100 column chromatography.

RNase Tf-3 and RNase Tf-4 were respectively found to have their optimum pH at 4.75 and 4.9 and molecular weights of approximately 13,000 and 16,000, as determined by gel filtration. Both enzymes were inhibited by Cu^{2+} and Hg^{2+} , and inactivated by heating at over 50°C. By addition of yeast RNA to the two enzymes, however, their thermostabilities increased. The activities of the enzymes were stable in a pH range of 4.5 to 6.5. Like other plant RNases, RNase Tf-3 and RNase Tf-4 appeared to have no preference for base in RNA.

Extracts from many plant species contain enzymes capable of degrading nucleic acids, and their properties have already been studied.^{1~6)} But there have been few reports on RNase preparations from shrubby plant such as tea plant. It is interesting to compare shrubby plant RNases with herbaceous plant RNases.

Takino and Imagawa⁷⁾ observed the increase of 2'- and 3'-isomers of mononucleotides during the manufacturing of black tea, and speculated that ribonuclease in tea leaves liberates these 2'- and 3'- isomer of mononucleotides from tea leaf RNA during manufacturing. Ogutuga and Northcote^{8~9)} reported that the rate of caffeine formation in an autolysing tea callus could be increased by the addition of RNA and suggested that the purines derived from nucleic acid catabolism by ribonuclease were transformed into caffeine. But there has been no report on study of tea leaf ribonuclease to clarify such a metabolism of nucleic acid in tea plant.

This paper reports on partial purification and some properties of tea leaf ribonucleases.

MATERIALS AND METHODS

Tea leaves. The first three leaves and a bud from new tea shoots (*Cameria sinensis*, clone *Yabukita*) were plucked at the field of the National Research Institute of Tea in May, and used for experiment.

Assay for ribonuclease. The assay for ribonuclease was based on the release of acid-soluble nucleotides from yeast RNA. The reaction mixture contained 0.6 ml of 0.05 M phosphate buffer, pH 5.5, 0.6 ml of 0.5% RNA solution, 0.4 ml of water and enzyme solution to give a final volume of 2 ml. The reaction was carried out at 37°C for 1 hr, and terminated by adding 0.4 ml of 25% perchloric acid, containing 0.75% uranyl acetate. After centrifugation at 2,500 rpm for 10 min, the supernatant was diluted with water 50-fold, and the optical density at 260 nm was measured. One unit of ribonuclease activity was defined as the amount of enzyme that causes an increase in absorbance of 1.00 at 260 nm in a volume of 1.0 ml.¹⁰⁾

Assay for protein. The amount of protein was measured by Nessler's method.¹¹⁾

DEAE-Cellulose column chromatography. A column (2×35 cm) was filled with activated DEAE-cellulose (Brown, Seikagaku Kōgyō) and was equilibrated with 0.02 M phosphate buffer, pH 6.6. A linear gradient elution was carried out with potassium chloride in the range of 0 to 0.4 M in the 0.02 M phosphate buffer, pH 6.0, after elution with 75 ml of the same buffer. Each eluate of 5 g was collected at a flow rate of 0.33 g/min.

Gel filtration on Sephadex G-100. Sephadex G-100

[†] This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan at Sapporo, July, 1975.

allowed to swell in 0.02 M phosphate buffer, pH 6.0, for 1 day was packed into a column (3×48 cm) and equilibrated with the same buffer. Each eluate of 5 g was collected at a flow rate of 0.42 g/min.

Estimation of molecular weight. Molecular weight of the enzyme was estimated by Sephadex G-100 column chromatography.¹²⁾ The column mentioned above was calibrated for molecular weight estimation with cytochrome C: 12,400, chymotrypsinogen: 25,000 and ovalbumin: 45,000.

Column chromatography of digested RNA and acid-soluble fraction. Three different solutions were applied to Liquid Chromatograph (Nihon Denshi Co., Ltd. JLC 6-US) whose column (0.8×11 cm) was packed with anion exchange resin LCR-3.

Exhaustively digested RNA solution: 1% yeast RNA solution in phosphate buffer, pH 5.0, was digested with tea leaf RNases at 37°C for 18 hr.

Acid-soluble fraction (1): Acid-soluble fraction was separated by adding 25% perchloric acid, from 1% RNA digested in phosphate buffer, pH 5.0, with tea leaf RNases at 37°C for 1 hr, and then adjusted to pH 6.5 with 2.5 N potassium hydroxide.

Acid-soluble fraction (2): Acid soluble fraction (2) was obtained by hydrolyzing acid-soluble fraction (1) with 0.5 N potassium hydroxide at 37°C for 18 hr, and adjusted to pH 6.5 with 25% perchloric acid. These samples were then eluted stepwise with 2.5×10^{-3} N HCl for 40 min, 4×10^{-3} N HCl+0.03 M NaCl for 25 min, and 10^{-2} N HCl+0.1 M NaCl for 25 min at a flow rate of 1.3 ml/min at 50°C.

RESULTS

Purification and isolation of ribonuclease from tea leaves

One kg of tea leaves was homogenized in 2 liters of 0.05 M phosphate buffer, pH 7.0, with 200 g of polyclar AT (polyvinyl pyrrolidone). The homogenate was filtrated with 4 sheets of gauze and centrifuged at 10,000 rpm for 20 min, and the precipitate was discarded. 1700 ml of clear supernatant was obtained. 500 ml of supernatant was used for the next procedure, and the rest was stored frozen at -15°C.

A 500 ml portion of supernatant solution was brought to 30% saturation by gradual addition of solid ammonium sulfate. After standing for 1 hr, the suspension was centrifuged at 10,000 rpm for 15 min, and the precipitate was discarded. Then solid ammonium

sulfate was further added to 70% saturation, and after 3 hr the precipitate obtained by centrifugation at 10,000 rpm for 15 min was dissolved in 0.02 M phosphate buffer, pH 5.5. The solute was dialyzed against the same buffer, and 57 ml of dialyzate was obtained.

All of the dialyzate was applied on a DEAE-cellulose column and the linear gradient elution was carried out with potassium chloride. As shown in Fig. 1, four fractions containing RNase activity were separated by the DEAE-cellulose column chromatography, and they were designated RNase Tf-1, RNase Tf-2, RNase Tf-3 and RNase Tf-4 in the order of elution time.

FIG. 1. Column Chromatography of the Crude Enzyme on DEAE-Cellulose.

●, enzyme activity; △, absorbance at 280 nm of elute; ×, Cl^- concentration of elute.

(1) RNase Tf-1; (2) RNase Tf-2; (3) RNase Tf-3; (4) RNase Tf-4.

The two bigger fractions of RNase Tf-3 and RNase Tf-4 in tea leaf RNase were packed in visking tube and condensed to 10 ml with solid polyethylen glycol 6000. Then they were applied on a Sephadex G-100 column, and eluted out with the same buffer. As shown in Figs. 2 and 3, RNase Tf-3 and RNase Tf-4 were eluted as peaks of fraction number 45~60 and fraction number 44~58, respectively.

The process of purification of the enzyme is summarized in Table I. The specific activity of RNase Tf-3 increased 223.5-fold after Sephadex G-100 column chromatography and RNase Tf-4 782-fold. The recoveries of the two activities were 4.3% and 32.6% of the original activities, respectively.

FIG. 2. Sephadex G-100 Column Chromatography of RNase Tf-3.

○, absorbance at 280 nm of elute; ●, enzyme activity.

FIG. 3. Sephadex G-100 Column Chromatography of RNase Tf-4.

○ and ● represent the same as in Fig. 2.

Properties of RNase Tf-3 and RNase Tf-4

Effect of pH on the activity. The pH activity curves on RNase Tf-3 and RNase

Tf-4 shown in Fig. 4 indicate the optimum pH of RNase Tf-3 to be 4.75 and that of RNase Tf-4, about 4.9.

FIG. 4. pH-activity Curves on RNase Tf-3 and RNase Tf-4.

●, RNase Tf-3; ○, RNase Tf-4.

pH stability. The two enzyme solutions were kept at different pH levels at 4°C overnight and their activities were assayed. RNase Tf-3 and RNase Tf-4 were stable in the range of pH 4.0~6.5. (Fig. 5)

Effect of temperature on the activity. The temperature-activity curves on RNase Tf-3 and

TABLE I. PURIFICATION OF TEA LEAF RIBONUCLEASES

	Volume (ml)	Enzymes (units) ^{a)}	Protein (mg)	Specific activity	Purification	Yield (%)
Crude extract	500	1166	2500	0.47	1.0	100
Salting out	48	919				78.8
Dialysis	57	908				77.9
DEAE-Cellulose chromatography						
RNase Tf-3	35	53	20.35	2.58	5.5	4.5
Fr. No. 98~104						
RNase Tf-4	135	386	30.53	12.64	27.1	33.1
Fr. No. 109~135						
Sephadex G-100 chromatography						
RNase Tf-3	65	50	0.48	104.17	223.5	4.3
RNase Tf-4	80	380	1.04	365.38	782.0	32.6

^{a)} Units/ml = $\frac{\text{O.D. at 260 nm} \times (\text{ml assay solution} + \text{ml precipitating agent})^{10})}{(\text{ml enzyme solution assayed}) \times \text{min}}$

FIG. 5. Effect of pH on the Activity of RNase Tf-3 and RNase Tf-4.

●, RNase Tf-3; ○, RNase Tf-4; —, acetate-sodium acetate buffer; ----, phosphate buffer.

RNase Tf-4 are shown in Fig. 6. The optimum temperature was 55°C for both RNase Tf-3 and RNase Tf-4.

FIG. 6. Temperature-activity Curves of RNase Tf-3 and RNase Tf-4.

●, RNase Tf-3; ○, RNase Tf-4.

Thermal stability. RNase Tf-3 and RNase Tf-4 in 0.02 M phosphate buffer, pH 6.0, were heated in water bath at different temperatures for 10 min and 30 min, and cooled immediately in ice water after the treatment. As shown in Fig. 7, RNase Tf-3 and RNase Tf-4 rapidly lost their activity above 50°C. However, when the two enzymes were heated in 0.01% RNA solution at 55°C for 10 min, the residual activity increased from 45% to 99% and from 60% to 88%, respectively, compared with those heated without RNA solution.

Effects of metal ions. As shown in Table

FIG. 7. Effect of Heating on the Activity of RNase Tf-3 and RNase Tf-4.

(1) RNase Tf-3; (2) RNase Tf-4.

●, 10 min; ○, 30 min.

II, the two enzymes were inhibited about 50% by 10^{-3} M Cu^{2+} and about 70% by 10^{-3} M Hg^{2+} . However, Mn^{2+} , Mg^{2+} , Ca^{2+} , and Zn^{2+} had no clear effects on the enzyme activity.

Effects of inhibitors. The effect of inhibitors including monoiodoacetic acid, para-chloromercuric benzoate, ethylene diamine tetraacetic acid (EDTA) and potassium cyanide were examined. As shown in Table II, no clear inhibition was observed.

Estimation of molecular weights. The molecular weights of RNase Tf-3 and RNase Tf-4, as determined by gel filtration, are given in Fig. 8. RNase Tf-3 and RNase Tf-4 have

FIG. 8. V_e/V_o -molecular Weight Relation of Standard Proteins, RNase Tf-3 and RNase Tf-4.

A, cytochrome C (12,400); B, chymotrypsinogen (25,000); C, ovalbumin (45,000); D, RNase Tf-3; E, RNase Tf-4.

TABLE II. EFFECTS OF INHIBITORS AND METAL IONS

Effects of Inhibitors			Effects of Metal Ions		
10 ⁻³ M	RNase Tf-3	RNase Tf-4	10 ⁻³ M	RNase Tf-3	RNase Tf-4
None	100 (%)	100 (%)	None	100 (%)	100 (%)
PCMB	104	105	CaCl ₂	99	103
Monoiodo			MgCl ₂	105	104
acetic acid	96	103	SnCl ₂	108	104
EDTA	83	76	FeCl ₂	103	100
KCN	106	110	CuSO ₄	50	52
Citric acid	99	104	ZnSO ₄	88	89
Oxalic acid	100	102	(AcO ⁻) ₂ Pb	107	109
ATP	86	91	(AcO ⁻) ₂ Hg	31	29
ADP	89	84			

molecular weights of about 13,000 and about 16,000, respectively.

Substrate specificity. Column chromatography of nucleotides digested from yeast RNA with RNase Tf-3 and RNase Tf-4 was performed to see how they attacked inter-nucleotide bonds in RNA.

At first, exhaustively digested yeast RNAs with RNase Tf-3 and RNase Tf-4 were chromatographed. Figure 9 shows RNase Tf-3 hydrolyzes yeast RNA almost completely into 3'-mononucleotides. But the hydrolyzate by RNase Tf-4 contained many other oligonucleotides, therefore individual mononucleotides could not be identified clearly.

Acid-soluble fractions were separated from the RNA digests, and chromatographed after neutralization. Figures 10-(a) and 11-(b) show cytidylic acid, adenylic acid, uridylic acid and guanylic acid were found in the acid-soluble fractions. But no peaks of uridylic acid and guanylic acid were clearly isolated, probably because the oligonucleotides, which are very similar to uridylic acid and guanylic acid in elution time, were still contained in the acid-soluble fractions.

So the acid-soluble fractions were hydrolyzed into mononucleotides by alkali at 37°C for 18 hr and chromatographed after neutralization. As shown in Fig. 10-(a') and 11-(b'), the peaks of seven nucleotides, 2'-cytidylic acid, 3'-cytidylic acid, 2'-adenylic acid, 3'-adenylic acid, 2'-and 3'-uridylic acid, 2'-

FIG. 9. Column Chromatography of Exhaustively Digested Yeast RNAs with RNase Tf-3 and RNase Tf-4.

(2) 3'-cytidylic acid; (4) 3'-adenylic acid; (5) 3'-uridylic acid; (7) 3'-guanylic acid.

A, Exhaustively digested RNA solution with RNase Tf-3; B, Exhaustively digested RNA solution with RNase Tf-4.

guanylic acid and 3'-guanylic acid, were clearly isolated, and therefore the amounts of these nucleotides could be determined. Table III shows all these mono nucleotides were liberated at approximately the same rate

FIG. 10. Column Chromatography of Nucleotides in Acid-soluble Fractions (1) and (2) Obtained from Yeast RNA Digested with RNase Tf-3.

1, 2'-cytidylic acid; 2, 3'-cytidylic acid; 3, 2'-adenylic acid; 4, 3'-adenylic acid; 5, 2'(3')-uridylic acid; 6, 2'-guanylic acid; 7, 3'-guanylic acid; 8, unknown (oligonucleotides).

FIG. 11. Column Chromatography of Nucleotides in Acid-soluble Fractions (1) and (2) Obtained from Yeast RNA Digested with RNase Tf-4.

1~8 represent the same as in Fig. 10.

in acid-soluble digests by RNase Tf-3 and RNase Tf-4 from yeast RNA.

DISCUSSION

RNase Tf-3 and RNase Tf-4 obtained from

TABLE III. LIBERATION RATES OF MONONUCLEOTIDES FROM YEAST RNA BY RNase Tf-3 AND RNase Tf-4

Each figure indicates the percentage of liberation of the corresponding mononucleotide from yeast RNA, and includes the combined total of 2'- and 3'-monophosphate.

Mono-nucleo-tides	RNase Tf-3		RNase Tf-4	
	Digestion time (min) 60	Digestion time (min) 120	Digestion time (min) 60	Digestion time (min) 120
CMP	19 (%)	46 (%)	9 (%)	59 (%)
UMP	40	54	24	62
AMP	23	44	15	59
GMP	37	57	24	78

tea leaves proved similar to other herbaceous plant RNase in several properties. The optimum pH of RNase Tf-3 and RNase Tf-4 were very close to the reported values of 5.1 for tobacco,¹³⁾ 5.0~5.8 for corn,²⁾ and 5.0 for spinach.¹⁴⁾

The two enzymes were not so thermally stable as tobacco leaf RNase,¹³⁾ spinach leaf RNase¹⁴⁾ and cucumber seedling RNase,¹⁵⁾ but their thermostability increased with the addition of yeast RNA.

Plant RNases, such as those derived from spinach,¹⁴⁾ soybean,¹⁶⁾ mungbean,¹⁷⁾ ryegrass¹⁸⁾ and barley,¹⁹⁾ catalyze the hydrolysis of RNA to liberate purine and pyrimidine mononucleotides as the final products. RNase Tf-3 and RNase Tf-4 were found similar type to these enzymes in that they are nonspecific for bases of substrate. As 2'- and 3'-isomers of mononucleotides were contained in acid-soluble nucleotides digested from yeast RNA with RNase Tf-3 and RNase Tf-4, it is thought that 2',3'-cyclic mononucleotides turned into 2'- and 3'-isomers of mononucleotides with the addition of stopping reagent. So it may be reasonable to think that RNase in tea leaves hydrolyze the RNA in tea leaves and increase these 2'- and 3'-isomers of mononucleotides during manufacturing of black tea. Final products from yeast RNA digested with RNase Tf-3 were 3'-mononucleotides, and those with RNase Tf-4 contained many oligonucleotides. These results suggest that the two enzymes are endonucleases.

REFERENCES

- 1) C. M. Wilson *Plant Physiol.*, **43**, 1332 (1968).
  - 2) C. L. Kado, *Arch. Biochem. Biophys.*, **125**, 86 (1968).
  - 3) O. J. Lantero and H. J. Klosterman, *Phytochemistry*, **22**, 775 (1973).
  - 4) L. Jervis, *ibid.*, **13**, 709 (1974).
  - 5) M. Irie, *Protein, Nucleic acid and Enzyme*, **12**, 386 (1967).
  - 6) E. A. Barnard, *Ann. Rev. Biochem.*, **38**, 677 (1969).
  - 7) Y. Takino and H. Imagawa, *Nippon Syokuhin Kogyô Kaishi*, **20**, 143 (1973).
  - 8) D. B. A. Ogutuga and D. H. Northcote, *Biochem. J.*, **117**, 715 (1970).
  - 9) D. B. A. Ogutuga and D. H. Northcote, *J. Exp. Botany*, **21**, 258 (1970).
  - 10) C. M. Wilson, *Ann. Rev. Plant Physiol.*, **26**, 187 (1975).
  - 11) J. F. Thompson and G. R. Morrison, *Anal. Chem.*, **23**, 1153 (1951).
  - 12) P. Andrews, *Biochem. J.*, **91**, 222 (1964).
  - 13) W. F. Niggemeyer and K. K. Reddi, *Biochim. Biophys. Acta*, **26**, 40 (1959).
  - 14) C. M. Wilson, *ibid.*, **68**, 177 (1963).
  - 15) E. W. Tuve and C. B. Anfinsen, *J. Biol. Chem.*, **235**, 3437 (1960).
  - 16) A. J. Merola and F. F. Davis, *Biochim. Biophys. Acta*, **55**, 431 (1962).
  - 17) J. Stockx and L. Driessche, *Arch. Intern. Physiol. Biochim.*, **69**, 521 (1961).
  - 18) L. Shyster, H. G. Khorana and L. A. Heppel, *Biochim. Biophys. Acta*, **33**, 452 (1959).
  - 19) O. J. Lantero and H. J. Klosterman, *Phytochemistry*, **12**, 775 (1975).
-