STUDIES ON TOBACCO LEAF RIBONUCLEASE

II. MECHANISM OF ACTION*

K. K. REDDI

Virus Laboratory, University of California, Berkeley, Calif. (U.S.A.)

In a previous communication the purification and properties of ribonuclease occurring in tobacco leaves were reported[1]. The present communication deals with the mechanism of its action on ribonucleic acid. The results recorded here show that the tobacco leaf ribonuclease cleaves all the internucleotide linkages in ribonucleic acid giving rise to nucleoside 2′:3′-cyclic phosphates. The enzyme has no action on pyrimidine cyclic nucleotides while it slowly hydrolyses purine cyclic nucleotides to nucleoside 3′-phosphates exclusively.

EXPERIMENTAL

Tobacco leaf ribonuclease (TLRNase) was prepared from Turkish tobacco leaves according to the procedure of FRISCH-NIGGEMEYER AND REDDI[1]. Tobacco mosaic virus nucleic acid (TMV-NA) was prepared using the heat-denaturation method[2,3] with some modification[4]. Prostatic phosphomonoesterase was kindly supplied by Dr. MORRIS LONDON. Dried *Crotalus adamanteus* venom was obtained from Ross Allen's Reptile Institute, Silver Springs, Florida. Pancreatic ribonuclease was a crystalline preparation obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. The purine and pyrimidine bases, nucleotides and cyclic adenylic, uridylic and cytidylic acids were obtained from Schwartz Laboratories Inc., New York. The cyclic guanylic acid was prepared in the laboratory using the action of TLRNase on TMV-NA and the details of the procedure are given below.

Fractionation, identification and estimation of the products formed by hydrolysing TMV-NA with TLRNase

The reaction mixture, containing 2 mg TMV-NA in 0.4 ml of 0.1 *M* ammonium acetate buffer at pH 5.1, 200 units of TLRNase and a few drops of toluene, was incubated at 36° for 18 h. Four such complete digests were subjected to paper chromatography. Aliquots of 0.1 ml were placed in a band at a distance of 10 cm from the top of Whatman 3 mm filter paper (55 × 24 cm) and dried in a current of air. The chromatograms were developed for 24 h at room temperature in a solvent system containing isopropanol (700 ml), water (300 ml) and 0.35 ml NH_3 per liter of air space[5]. The developed chromatograms were dried at room temperature and Fig. 1 is a photograph taken according to the modified technique of SMITH AND ALLEN[6]. This procedure fractionated the hydrolysate into four distinct bands numbered 1 to 4 in order of R_F values (Fig. 1). The bands were cut out and eluted by allowing the distilled water to run along them. The eluates of each band from all the chromatograms were combined

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and concentrated *in vacuo* at room temperature. The chemical nature and the amounts of the substances present in each of the bands were established by using the following procedures.

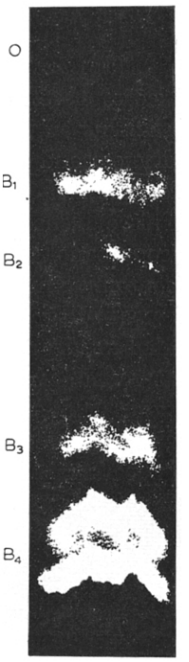

Fig. 1. Chromatogram of the hydrolysate obtained by the action of TLRNase on TMV-NA for 18 h at pH 5.1 and 36°, developed in isopropanol–water–NH_3 solvent system for 24 h at room temperature. O, origin of chromatogram; B 1, guanosine 3′-phosphate; B 2, adenosine 3′-phosphate; B 3, guanosine 2′,3′-cyclic phosphate; B 4, adenosine 2′,3′-cyclic phosphate, cytidine 2′,3′-cyclic phosphate and uridine 2′,3′-cyclic phosphate.

Band 1

(a) *Electrophoresis at pH 3.5.* An aliquot of the eluate was subjected to paper electrophoresis in 0.05 *M* ammonium formate buffer at pH 3.5[5]. Only one spot corresponding to guanylic acid was observed. The spot was eluted with the distilled water. The eluate was concentrated to dryness. The dried residue was hydrolysed with 70–72 % $HClO_4$ and the digest after diluting with equal amount of distilled water was subjected to paper chromatography in isopropanol–HCl–water solvent system[7] for 18 h at room temperature. One spot corresponding to guanine was observed on the chromatogram. This evidence and also the position of band 1 in the chromatogram developed in isopropanol–water–NH_3 solvent system suggest that band 1 contains guanosine 2′- or 3′- or 5′-phosphate or a mixture of all the three nucleotides.

(b) *Action of snake venom.* Reaction mixture consisting of eluate equivalent to 5 γ phosphorus in 0.5 ml of 0.05 *M* veronal buffer (pH 8.5) containing 0.01 *M* $MgSO_4$ and 100 γ snake venom was incubated at 37° for 2 h. No inorganic phosphorus was found. Adenosine 5′-phosphate when incubated under the same conditions gave rise to adenosine and inorganic phosphorus. Hence the band 1 does not contain guanosine 5′-phosphate.

(c) *Action of phosphomonoesterase.* Reaction mixture containing eluate equivalent to 5 γ phosphorus in 0.5 ml of 0.1 *M* sodium acetate buffer at pH 5.1 and 0.05 ml of phosphomonoesterase also in acetate buffer, was incubated at 37° for 2 h. All of the phosphorus was found as inorganic phosphorus. Hence the band 1 contains guanosine 2′- or 3′-phosphate or a mixture of both the isomers.

(d) *Chromatography in $(NH_4)_2SO_4$–isopropanol–sodium acetate solvent system.* An aliquot of the eluate was placed on Whatman No. 1 filter paper and developed in a solvent system containing saturated $(NH_4)_2SO_4$ in water–isopropanol–*M* sodium acetate (80:2:18)[5]. One spot corresponding to guanosine 3′-phosphate was observed.

All the above data go to show that band 1 contains guanosine 3′-phosphate only.

Band 2

By using the same procedures used for the identification of the substance in band 1, evidence was obtained to show that band 2 contains only adenosine 3′-phosphate.

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Band 3

(a) *Electrophoresis at pH 3.5.* An aliquot of the eluate was subjected to paper electrophoresis at pH 3.5 in the manner described above. One spot corresponding to guanylic acid was observed. The spot was eluted, the eluate was concentrated to dryness and hydrolysed with 70–72 % $HClO_4$. The chromatogram of the digest in isopropanol–HCl–water solvent system showed one spot corresponding to guanine. This evidence and also the position of band 3 in the chromatogram developed in isopropanol–water–NH_3 solvent system suggest that band 3 represents a guanine-containing nucleotide other than guanosine 2′- or 3′- or 5′-phosphate.

(b) *Action of 0.1 N HCl.* To an aliquot of the eluate, *N* HCl was added to bring the final concentration of the acid in the total mixture to 0.1 *N*. The mixture after holding at room temperature for 4 h, was subjected to chromatography in isopropanol–water–NH_3 solvent system. The spot corresponding to a mixture of guanosine 2′- and 3′-phosphates was observed on the developed chromatogram. The substance in band 3 appears to be guanosine 2′,3′-cyclic phosphate.

(c) *Action of phosphomonoesterase.* Conditions for hydrolysis were the same as described above. No inorganic phosphorus was released. However, the eluate which was treated with 0.1 *N* HCl at room temperature for 4 h was acted upon by the enzyme and all of the phosphorus was found in inorganic form.

The above evidence goes to show that band 3 contains guanosine 2′,3′-cyclic phosphate.

Band 4

(a) *Electrophoresis at pH 3.5.* Three spots corresponding to cytidylic acid, adenylic acid and uridylic acid were noticed. These were eluted separately, concentrated and hydrolysed with 70–72 % $HClO_4$ as described above. In the chromatogram of the digests in isopropanol–HCl–water solvent system, spots corresponding to cytosine, adenine and uracil were observed. This evidence and the position of band 4 in isopropanol–water–NH_3 solvent system suggest that in band 4 there are three nucleotides containing adenine, cytosine and uracil. The following experiments were done to establish their identity.

(b) *Action of 0.1 N HCl.* The experimental conditions were the same as described above. The acid-treated eluate when chromatographed in isopropanol–water–ammonia solvent system occupied the same position on the chromatogram as a mixture of corresponding noncyclic nucleotides.

(c) *Action of phosphomonoesterase.* Phosphomonoesterase did not have any action on the eluate. The eluate after treatment with 0.1 *N* HCl for 4 h at room temperature was acted upon by the enzyme and all of the phosphorus appeared in inorganic form.

(d) *Action of pancreatic ribonuclease.* 0.2 ml of 0.1 *M* borate buffer at pH 7.6 containing eluate equivalent to 10 γ phosphorus and 20 γ pancreatic ribonuclease was incubated at 36° for 18 h. The digest was subjected to chromatography in the isopropanol–water–NH_3 solvent system. The developed chromatogram showed two distinct bands; one occupied the same position as that of the reference spot containing cytidylic acid and uridylic acid and the other that of cyclic adenylic acid. Pancreatic ribonuclease is known to hydrolyse slowly the pyrimidine cyclic nucleotides while the purine cyclic nucleotides are unaffected[5].

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The above results show that band 4 contains the 2',3'-cyclic phosphates of adenosine, cytidine and uridine.

The amounts of the nucleotides present in the bands were determined spectrophotometrically and the results are given in Table I.

TABLE I

CHEMICAL NATURE AND THE AMOUNTS OF SUBSTANCES PRESENT IN EACH OF THE BANDS IN THE CHROMATOGRAM OF THE DIGEST OBTAINED BY THE ACTION OF TLRNASE ON TMV-NA FOR 18 h AT 36° AND pH 5.1

Chromatogram was developed in isopropanol–water–NH_3 solvent system[5]

Band. No. (Fig. 1)	*Nature of the substance*	*% of total GA in TMV-NA*	*% of total AA in TMV-NA*	*% of total CA in TMV-NA*	*% of total UA in TMV-NA*
1	Guanosine 3'-phosphate	40.0			
2	Adenosine 3'-phosphate		12.3		
3	Guanosine 2',3'-cyclic phosphate	60.0			
4	Adenosine 2',3'-cyclic phosphate		87.7		
	Cytidine 2',3'-cyclic phosphate			100	
	Uridine 2',3'-cyclic phosphate				100

GA = guanylic acid; AA = adenylic acid; CA = cytidylic acid; UA = uridylic acid; TMV-NA = tobacco mosaic virus nucleic acid.

Base composition of dialysable fragments formed during the degradation of TMV-NA with TLRNase

Reaction mixture containing 5 mg TMV-NA in 0.5 ml of 0.1 *M* ammonium acetate buffer at pH 5.1 and 120 units of TLRNase was dialysed at 36° against 4 ml of 0.1 *M* acetate buffer at pH 5.1. At definite intervals of time (Fig. 2) the outside dialysate was replaced with a fresh amount of 4 ml acetate buffer. The dialysable material was concentrated to dryness. The residue was hydrolysed with 72 % $HClO_4$ and the base composition was determined[7]. These results are given in Fig. 2.

Action of TLRNase on cyclic nucleotides

The reaction mixture containing 500 γ cyclic nucleotides in 0.2 ml of 0.1 *M* ammonium acetate buffer at pH 5.1 and 120 units of TLRNase was incubated at 36° for 18 h. Suitable blanks were run side by side. An aliquot was placed on Whatman No. 3 filter paper and developed in the isopropanol–water–ammonia solvent system for 24 h at room temperature. The dried chromatogram was viewed under ultraviolet light, the spots were marked with a pencil and eluted with 0.01 *N* HCl. Their amounts were determined spectrophotometrically. The results are given in Table II.

RESULTS AND DISCUSSION

The hydrolysate of TMV-NA obtained by the action TLRNase for 18 h at 36° (pH 5.1) when subjected to chromatography in isopropanol–water–NH_3 solvent system,

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TABLE II

HYDROLYSIS OF CYCLIC NUCLEOTIDES BY TLRNASE FOR 18 H AT pH 5.1 AND 37°

Cyclic nucleotides	*% hydrolysed to corresponding nucleoside 3′-phosphate*
Guanosine 2′,3′-phosphate	21.2
Adenosine 2′,3′-phosphate	10.5
Cytidine 2′,3′-phosphate	0
Uridine 2′,3′-phosphate	0

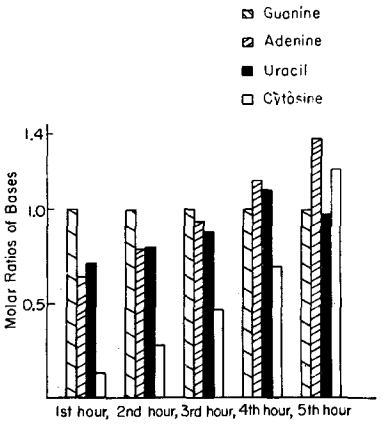

Fig. 2. Composition of dialysable material formed during the course of degradation of TMV-NA by TLRNase at pH 5.1 and 36°.

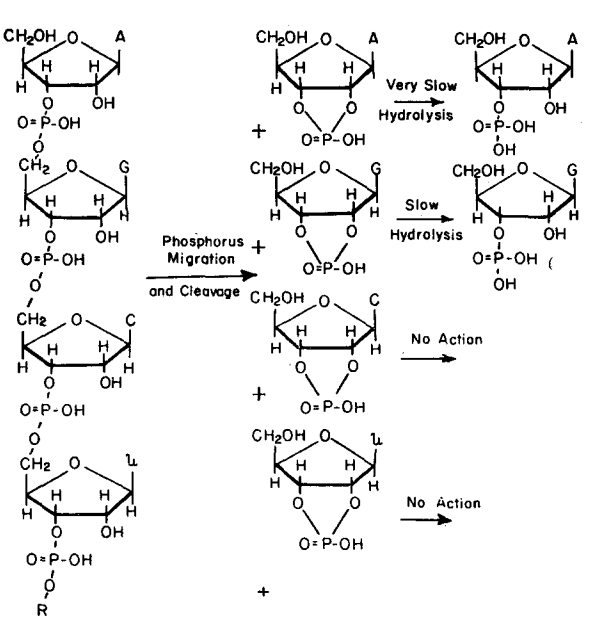

Fig. 3. The action of TLRNase on a polyribonucleotide. A = adenine; G = guanine; C = cytosine; U = uracil; R = rest of the chain.

resulted in the separation of 4 distinct bands (Fig. 1). Band 1 contains guanosine 3′-phosphate; band 2, adenosine 3′-phosphate; band 3, guanosine 2′,3′-cyclic phosphate and band 4, a mixture of adenosine 2′,3′-cyclic phosphate, cytidine 2′,3′-cyclic phosphate and uridine 2′,3′-cyclic phosphate (Table I). The TLRNase brings about complete disruption of all the internucleotide linkages in ribonucleic acid to give rise to nucleoside 2′,3′-phosphates of both purines and pyrimidines. The purine cyclic nucleotides are slowly hydrolysed to nucleoside 3′-phosphates exclusively while the pyrimidine cyclic nucleotides are resistant to further action of the enzyme. The enzymic cleavage proceeds via intramolecular transphosphorylation followed by hydrolysis in the case of the purine nucleoside cyclic phosphates. The mechanism of its action is summarized in Fig. 3. The action of TLRNase differs from that of pancreatic ribonuclease which hydrolyses only secondary phosphate esters of pyrimidine ribonucleoside 3′-phosphates[5,8,9]. The mode of action of this enzyme appears to resemble to some extent that of the enzyme in pea leaves[10,11].

Even though the TLRNase brings about the cleavage of all the diester bonds in ribonucleic acid, at least in the initial stages of digestion it appears to favour certain

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bonds; thus during the first hour of digestion the dialysable fragments are rich in guanine and poor in cytosine (Fig. 2). These results indicate that the enzyme has much more preference for secondary phosphate esters of guanosine 3'-phosphates and much less preference for secondary phosphate esters of cytidine 3'-phosphates. However, given time all the diester bonds are completely broken down by the enzyme.

The results presented in Table II show that TLRNase slowly hydrolyses purine cyclic nucleotides while the pyrimidine cyclic nucleotides are inert to its further action. Of the two purine cyclic nucleotides, adenosine 2',3'-phosphate is hydrolysed much more slowly than guanosine 2',3'-phosphate.

The TLRNase can be used with advantage to prepare both purine and pyrimidine cyclic nucleotides. It can also be used for determining the pyrimidine nucleoside 2'- or 3'-phosphate endings in a polyribonucleotide.

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SUMMARY

The mode of action of tobacco leaf ribonuclease on ribonucleic acid has been studied. Unlike the pancreatic ribonuclease this enzyme cleaves all the diester bonds and gives rise to purine and pyrimidine cyclic nucleotides. While the pyrimidine cyclic nucleotides are inert to further action of this enzyme, the purine cyclic nucleotides are slowly hydrolysed to nucleoside 3'-phosphates exclusively. Even though the enzyme disrupts all the internucleotide linkages in the ribonucleic acid it appears to have much more preference in the initial stages of digestion for guanosine 3'-phosphate linkages and much less preference for cytidine 3'-phosphate linkages.

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