Studies on the Nucleases Produced by Microorganisms

Part III. Properties of Partially Purified Phosphodiesterase (5'-Nucleotide-Forming) Produced by *Pellicularia* sp^{*,**}

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The properties of crude phosphodiesterase forming 5'-mononucleotide of *Pellicularia* H-11 were investigated on its metal requirement, pH response for activity and so on. The dialyzate of crude PDase against distilled water became partly inactive, but was recovered with Zn⁺⁺, Mn⁺⁺ and Mg⁺⁺, whereas completely inactivated dialyzate against EDTA was restored specifically with only Zn⁺⁺.

The optimum pH of PDase activity was 5.0 and that of ribonuclease 4.0. The crude PDase was partially purified by acetone fractionation and Amberlite IRC-50 (XE-64) or CM-cellulose column chromatography. Two PDase and a RNase activities were recognized.

Pellicularia PDase was found to be of new type according to its Zn⁺⁺ dependency and non-activity towards bis-*p*-nitrophenyl phosphate.

INTRODUCTION

Certain nucleases of different specificities are used for determination of nucleotide arrangements in nucleic acids. (Pancreatic RNase: EC 2.7.7.16, RNase T₁: EC 3.1.4.8, snake venom PDase.) And there have been found several nucleases having different properties or specificities in many organisms. Many of them are those hydrolyzing RNA or DNA to 3'-mononucleotides or oligonucleotides having 3'-terminal phosphate.¹⁻¹⁰⁾ Be-

sides, nucleases hydrolyzing RNA or DNA to 5'-mononucleotides or oligonucleotides having 5'-terminal phosphate have been found in *E. coli*,¹¹⁻¹³⁾ *Azotobacter agilis*,^{14,15)} *Penicillium*,¹⁶⁾

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** The following abbreviations are used: PDase, phosphodiesterase; RNase, ribonuclease; bis-*p*-NPP, bis-*p*-nitrophenyl phosphate.

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CHART

Asterococcus,¹⁷⁾ *Rhodotorula*¹⁸⁾ and so on.

It does not need many words to mention that the PDase-producing microorganism, which can be cultivated in a large scale, is useful for fermentation industry, because 5'-IMP and 5'-GMP are good seasoning for Japanese food.

A new type of nuclease would contribute to investigation of a primary structure in nucleic acids. The screening of microorganism producing activity of PDase as high as possible has been carried out already in the previous reports, using about three hundred strains of Phycomycetes, Ascomycetes, Basidiomycetes and Fungi Imperfecti. PDase activity of *Pellicularia* H-11 strain was the highest.

The procedures in cultivation and some properties of this PDase were also previously

reported.^{19,20)} This paper describes some properties and substrate specificities of crude and partially purified PDases.

The enzyme was found to be of new type because of its Zn^{++} dependency and non-activity towards bis-*p*-NPP.

MATERIALS AND METHODS

Strain and Culture Medium. *Pellicularia* H-11 and the following medium were used: glucose 5%, Mikuni meat extract 0.25%, Polypepton 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.05%, and $ZnSO_4 \cdot 7H_2O$ 0.1% (pH 6.0).

Assay of the PDase Activity (Activity at 660 m μ). 0.2 ml of 5% yeast RNA solution (purchased from N.B.C.) was added to a mixture of 1.0 ml of 0.026 M veronal acetate buffer (pH 6.0) and an appropriate amount of the enzyme solution. Water was added to the mixture up to the final volume, 2.0 ml. The reaction mixture was incubated at 60°C for 10 min., and then immersed into a boiling water for 5 min. to stop the reaction. 0.5 ml of the above was then incubated at 37°C for 2 hrs., with 0.5 ml of snake (*T. flavidis*) venom 5'-nucleotidase obtained by Butler's method²¹⁾ and 1.0 ml of 0.05 M Tris-HCl buffer (pH 8.5) containing 2×10^{-3} M $MgCl_2$. Inorganic phosphate was determined by the Allen's method,²²⁾ 0.5 ml having been withdrawn from the above fluid thereafter. Thus, the difference of the amount of inorganic phosphate between the diesterase-digested solution and its 5'-nucleotidase-treated solution accounted for true inorganic phosphate liberated from 5'-mononucleotides produced by the PDase action upon the RNA. As was mentioned in the former paper,^{19,20)} Zn^{++} was removed by Amberlite IR-120, prior to 5'-nucleotidase treatment in order to prevent Zn^{++} -inhibition of the enzyme activity, when the reaction mixture contained excess Zn^{++} .

Assay of RNase Activity by Determination of Ultraviolet Light Absorption in Acid-Soluble Materials (Activity at 260 m μ). The same reaction mixture as above assay was incubated at 37°C for

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30 min. or at 60°C for 15 min., and then chilled in an ice-water bath for 5 min., to which 2.0 ml of 0.25% uranium acetate-2.5% perchloric acid reagent²³⁾ was added. The mixture was kept at 0°C for 5 min., centrifuged for 10 min. at 800×g and the supernatant was diluted 100 times with distilled water. Its optical density was measured at 260 mμ.

PDase Preparation. After cultivated in 100 ml media of 500-ml Sakaguchi flask on reciprocal shaker at 30°C, 7 days, crude enzyme was obtained in the supernatant of the culture fluid, the cells having been centrifuged. Partially purified enzyme was prepared by the following procedure. Centrifuged supernatant was concentrated to about one fifth volume in vacuo at 40°C, to which, at -8°C, cold acetone was slowly added in the cold room (0~5°C) with gentle stirring until the concentration reached to 55% (v/v). After being kept at 0°C for 30 min., the brown precipitate formed (P-I) was centrifuged for 10 min. at 10,000×g. Cold acetone was added to the supernatant until 80% (v/v) concentration was reached. After 30 min. the precipitate (P-II) was obtained by centrifuging for 10 min. at 10,000×g. The acetone concentration of supernatant was then raised up to 90% (v/v) and the precipitate (P-III) was reformed. Only P-II fraction contained PDase activity.

CM-Cellulose Column Chromatography. CM-Cellulose column was prepared according to Peterson and Sober²⁴⁾ from Toyo Roshi cellulose powder (100~200 mesh). The column (1.3×28.5 cm) was equilibrated with 0.005 M Acetate buffer (pH 5.8). The fraction P-II was dissolved into a small amount of 0.005 M same buffer and dialyzed overnight against the same buffer. The dialyzate (O.D. at 280 mμ: 69, O.D. at 660 mμ: 200) was applied on the top of the column and eluted at a rate of 20 ml per hr with a concave gradient by 310 ml of 0.005 M Acetate buffer (pH 5.8) in mixing vessel and 190 ml of the same buffer of 0.5 M in the reservoir. The fraction volume was 5 ml. Fig. 5 shows the pattern obtained.

Amberlite IRC-50 (XE-64) Column Chromatography. The above dialyzate (O.D. at 280 mμ: 55.2, O.D. at 660 mμ: 160, O.D. at 260 mμ: 13,2000) was charged on to Amberlite XE-64 column (1×45 cm) previously bufferized with 0.005 M acetate buffer (pH 5.8). The column was washed with the same

buffer and then eluted with the same gradient system as used for CM-cellulose column chromatography at a rate of 40 ml per hr. (Fig. 6). The fraction volume was 10 ml.

Activity towards bis-*p*-NPP of PDase. Reaction mixture (3.0 ml) consisted of 1.0 ml of 0.005 M Na-bis-*p*-NPP, 1.0 ml of 0.026 M veronal acetate buffer (pH 5.0) or 0.01 M Tris-HCl buffer (pH 8.5) and 1.0 ml of the enzyme solution. The mixture, in which enzyme was replaced with water, was taken as blank. After incubation at 37°C for 2 hrs., 1.0 ml of 1 N NaOH was added to the reaction. The amount of *p*-nitrophenol, which was liberated if the PDase cleaved the phosphate linkage of the substrate, was estimated by the O.D. at 430 mμ.

Paper Chromatography of RNA Digests by PDase. RNA digests by the crude and the partially purified PDase were spotted on Toyo Roshi No. 51 and developed either by MeOH (50)-EtOH (25)-conc. HCl (6)-H₂O (19)²⁵⁾ or two dimensionally first by EtOH (75)-1 M CH₃COONH₄, pH 7.5 (30), and then, by saturated (NH₄)₂SO₄ (80)-H₂O (2)-isopropanol (18).²⁶⁾ The U.V.-absorbing materials were detected as dark spots by Minera Light. 5'-Nucleotides were visible by the reaction of sugar moieties with Schiff's reagent after periodate oxidation. They were also demonstrated by detecting organic phosphate with Hanes-Isherwood's reagent.

Effect of Zn⁺⁺ on Dialyzed Enzyme. An appropriate amount of ZnSO₄·7H₂O (0~17×10⁻⁴ M, final concn.) was added to the PDase assay mixture containing 0.1 ml of dialyzed enzyme.

RESULTS

RNA Digestion by Crude PDase

About 80% of RNA was hydrolyzed by the crude preparation of *Pellicularia*, basing upon the increase in inorganic phosphate liberated and in UV absorption in reaction mixture. The digest contained 5'-mononucleotides in 74% equivalent amounts of RNA used. (Fig. 1).

Effect of Zn⁺⁺ on Dialyzate of Crude PDase

The culture fluid was dialyzed against distilled water for 24 hrs. (H₂O-dialyzate).

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FIG. 1. Hydrolysis of RNA by PDase.

The reaction mixture, containing 0.2 ml of 5% RNA, 1.0 ml of veronal buffer (pH 6.0), 0.1 ml of culture fluid and 0.7 ml of H_2O , was incubated at $60^\circ C$.

FIG. 2. Effect of Zn^{++} on PDase of H_2O - and EDTA-Dialyzed.

An appropriate amount of $ZnSO_4 \cdot 7H_2O$ ($0 \sim 1.7 \times 10^{-2} M$) was added to the dialyzed PDase.

- : EDTA-dialyzed
- : H_2O -dialyzed
- ▲: Nondialyzed PDase

Also the culture fluid was dialyzed against 0.01 M EDTA for 24 hrs. and then against distilled water, which was frequently changed, for a week. (EDTA-dialyzed). When crude PDase was dialyzed against distilled water, the activity was partly lost, and, when against 0.01 M EDTA, it was completely inactivated. Both the partial and the complete inactivation could be perfectly recovered when a small amount of Zn^{++} was added. The optimum Zn^{++} concentration for recovery was $5.1 \times$

TABLE I. EFFECT OF METAL IONS ON H_2O -DIALYZATE AND EDTA-DIALYZATE

Metal	PDase Activity (O.D. at 660 mμ)	
	H_2O -Dialyzed	EDTA-Dialyzed
None	0.210	0.010
Ni^{++}	0.133	0.034
Co^{++}	0.095	0.047
Cu^{++}	0.100	0.010
Ca^{++}	0.121	0.025
Mg^{++}	0.274	0.002
Mn^{++}	0.300	0.002
Zn^{++}	0.258	0.210

Reaction mixture: 5% RNA, 0.2 ml; Veronal buffer, pH 6.0, 1.6 ml; Metal ions, 0.1 ml, Final concn. $1.7 \times 10^{-4} M$; Enzyme, 0.1 ml.

$10^{-4} M$ in H_2O -dialyzed and $10.2 \times 10^{-4} M$ in EDTA-dialyzed as shown in Fig. 2.

Effect of Several Metal Ions on H_2O - and EDTA-Dialyzed. As the influence of Zn^{++} on the activity was found, the effects of other metal ions were examined on the dialyzed as described in Table I. The original activity of enzyme, O.D. 0.34 in 660 mμ, was inactivated down to 0.21 after H_2O -dialysis, but Zn^{++} , Mg^{++} and Mn^{++} restored its activity up to 0.26, 0.27 and 0.30, respectively. Ni^{++} , Co^{++} , Ca^{++} and Cu^{++} inhibited the activity of H_2O -dialyzed, whereas the completely inactivated EDTA-dialyzed PDase was specifically recovered up to the original in $1.7 \times 10^{-4} M$ by only Zn^{++} .

On the basis of above results, it might be speculated that PDase in culture fluid includes two kinds of metals, the one is easily removable by dialysis, i.e. Mn^{++} , Mg^{++} and Zn^{++} as activator, and the other is the metal fraction at the active site which cannot be eliminated until treated by chelating agent like EDTA.

Comparison of PDase Activity (O.D. at 660 mμ) and RNase Activity (O.D. at 260 mμ) responding to pH changes. PDase activity and RNase activity were examined at pH 3 through pH 9 (Veronal buffer was used for pH 3 through 6 and Tris buffer for pH 7 through 9). The conditions of RNA hydrolysis were $60^\circ C$ -15 min

FIG. 3. Comparison of PDase Activity and RNase Activity at various pH values.

—△— : O.D. at 260 $m\mu$
 —○— : O.D. at 260 $m\mu$
 after { 60°C-15 min. reaction.
 37°C-30 min. reaction.
 —▲— : O.D. at 660 $m\mu$
 —●— : O.D. at 660 $m\mu$
 after { 60°C-15 min. reaction.
 37°C-30 min. reaction.

and 37°C-30 min. The optimal pH of PDase was 5.0, whereas that of RNase was 4.0 under both conditions (Fig. 3).

Furthermore, being shown in Table II, the fact was found that the degree in RNA hydrolysis and 5'-nucleotides formation exhibited discrepancy at every pH, and that ratios of 5'-nucleotides formed per RNA hydrolyzed were not constant at each pH.

More than half of RNA hydrolyzed was UV-absorbing materials besides 5'-mononucleotides during the comparatively early period of 60°C-15 min. and 37°C-30 min. reaction. After RNA digestion was sufficiently carried out at 60°C for 4 to 6 hr., 5'-mononucleotides occupied the most of RNA hydrolyzed (Fig. 1). These experiments indicate that the *Pellicularia* produces another RNase besides PDase.

TABLE II. RNA HYDROLYSIS AND 5'-NUCLEOTIDES FORMATION AT VARIOUS pH VALUES

pH	RNA Hydrolyzed % (a)*	5'-Nucleotides Formed % (b)**	(b)/(a)
60°C, 15 Min.			
Reaction			
3	37.5	5.3	0.141
4	75.0	26.6	0.354
5	65.2	32.0	0.490
6	56.5	26.6	0.471
7	52.1	20.3	0.389
8	37.5	7.3	0.194
37°C			
30 Min.			
Reaction			
3	32.0	7.2	0.225
4	48.9	16.6	0.339
5	44.0	18.0	0.409
6	40.2	16.4	0.407
7	35.3	13.0	0.368
8	25.3	4.6	0.184
9	20.6	2.0	0.097

* Acid-soluble materials obtained from KOH hydrolysis of RNA was calculated as 100%.

** Total Pi obtained from PCA hydrolysis of RNA was calculated as 100%.

Paper Chromatography of RNA Digested by Crude or Partially Purified Preparations. RNA hydrolyzed by the crude and partially purified enzymes (tube number 55 and 56 of Fig. 6) at 60°C for 40 min. (pH 5.0) was spotted on Toyo Roshi No. 51, and developed by ascending or descending solvent systems as shown in the Methods (Fig. 4). Both RNA hydrolyzates consisted mainly of four kinds of 5'-nucleotide. (AMP, GMP, CMP and UMP). At least three unknown spots and RNA core were shown on the chromatogram, which may be considered as nucleosides and oligonucleotides, respectively.

Purification by Column Chromatography

CM-Cellulose Column Chromatography. The acetone fraction, P-II, was chromatographed on CM-cellulose (Fig. 5). First peak was brown-colored impure proteins without activity. Second peak had PDase activity. Two PDase activities were found, which were activated by Zn^{++} .

FIG. 4. Paper Chromatography of RNA Digested by Partially Purified Enzyme.

- (a) One dimensional system, EtOH (50 v/v)-MeOH(25)-Conc. HCl(6)-H₂O(19); I: Enzyme used was acetone P-II fraction or Amberlite XE-64 number 55 fraction; II. Standard.
 (b) Two dimensional system, 1st: EtOH(75 v/v)-1 M CH₃COONH₄, pH 7.5 (30); 2nd: saturated (NH₄)₂SO₄(80)-H₂O(2)-isopropanol (18); Enzyme used was Amberlite XE-64 fraction number, 55 or 56.

FIG. 5. Column Chromatography of Acetone P-II Fraction on CM-cellulose.

Total O.D. at 280 mμ (—●—), 69
 Total O.D. at 660 mμ (—○—), 200
 O.D. at 660 mμ in Zn addition (---▲---)
 Column size, 1.3 cm × 28.5 cm; Gradient elution with 0.005 M Acetate buffer (pH 5.8) and same buffer of 0.5 M.

Amberlite XE-64 Column Chromatography.

Several peaks were separated by Amberlite XE-64 column chromatography (Fig. 6). Two PDases (I, II) were separated into peaks III and IV, respectively. Maximal activity of

PDase I was in fraction number 53 and that of PDase II in fraction number 56.

When RNase activity was examined, it came in number 55 between two PDase peaks. Therefore, another kind of RNase might be also contained in this fraction, on which we refer later in discussion.

The specific activity of each step in purification was described in Table V. There was no loss in activity throughout the procedure of concentration. The acetone fractionation (P-II) increased specific activity of PDase by about five times of the initial, accompanying the yield of less than a half. Although CM-cellulose did not display so much effect in purification procedure, in case of Amberlite XE-64 column chromatography, fraction number 55 was purified by 50-fold in PDase activity and 120-fold in RNase activity. (Table III).

The yield of PDase in this chromatography was approximately one third of P-II fraction. It was probably due to loss of activator of PDase (Zn⁺⁺, Mg⁺⁺ and Mn⁺⁺) that PDase yield decreased through acetone treatment and column chromatography. As shown in

Table IV, the addition of Zn^{++} to the partially purified fraction, in fact, increased its

activity, and the addition of EDTA resulted in complete inactivation of number 53.

This indicates that PDase of *Pellicularia* contains metal-activator fraction easily removable by dialysis, acetone fractionation and column chromatography, besides, that it has Zn^{++} -bound site, the metal of which cannot be taken away therefrom by such procedure except EDTA. Considering the above facts, the yield and specific activity of the purified preparation might be thought much higher, if, by adequate device, the metal deprival could be prevented.

TABLE III. PURIFICATION PROCEDURE OF *Pellicularia* PDASE

Culture fluid	
Concentration*	
Acetone fractionation	
Column chromatography	
CM-cellulose	
Amberlite XE-64	
*Concentrated solution	
Cold acetone 55% ($-8^{\circ}C$), kept standing for 30 min.	
then centrifuged at $10,000 \times g$ -10 min.	
Supernatant	Precipitate (P-I)**
Cold acetone 80% ($-8^{\circ}C$), kept standing for 30 min.	
then centrifuged at $10,000 \times g$ -10 min.	
Supernatant	Precipitate (P-II)**
Cold acetone 90% ($-8^{\circ}C$),	
centrifuged at $10,000 \times g$ -10 min.	
Supernatant	Precipitate (P-III)**
**P-I, P-II, P-III	
Dissolved respectively in Acetate buffer	
Dialysis overnight in cold room	

TABLE IV. EFFECT OF Zn^{++} AND EDTA

Enzyme	Zn^{++} ($1.7 \times 10^{-4} M$)	EDTA ($10^{-4} M$)	PDase activity (O.D. at 660 $m\mu$)
CM-cellulose tube No. 43	0	0	0.195
	1.0	0	0.235
	5.0	0	0.295
	10.0	5.0	0.225
Amberlite XE-64 tube No. 53	0	0	0.155
	0.3	0	0.185
	0.7	0	0.252
	0	5.0	0
	1.0	5.0	0
	5.0	5.0	0.278
	10.0	5.0	0.304

Reaction mixture: 5% RNA, 0.2 ml; 0.143 M Veronal buffer (pH 5.0), 1.0 ml; Zn^{++} , 0.1 ml; EDTA, 0.1 ml; Enzyme, 0.1 ml; H_2O , 0.5 ml.

TABLE V. SPECIFIC ACTIVITY IN PURIFICATION STEPS

Fractions	Protein O.D. at 280 $m\mu$	Yield %	PDase O.D. at 660 $m\mu$	Specific activity	RNase O.D. at 260 $m\mu$	Specific activity
Culture Fluid (500 ml)	1650	100	1000	1	180,000	1
Concentrated Soln. (100 ml)	1500	91	1050	1.1		
Acetone P-II (20 ml)	138	9.2	400	4.7	33,000	2.2
Amberlite XE-64						
Tube No. 53	0.80		9.0	18.4	23.5	10.8
54	0.75		1.5	3.3	65.0	32.1
55	0.30		10.1	55.8	100.0	122.5
56	0.85		19.9	38.6	33.0	13.5
57	0.90		5.7	10.3	12.0	4.9

FIG. 6. Column Chromatography of Acetone P-II Fraction on Amberlite XE-64.

{ Total O.D. at 280 $m\mu$, 55.2; Total O.D. at 260 $m\mu$, 13,200; Total O.D. at 660 $m\mu$, 160;
 (—●—) (—△—) (—○—)
 Column size, 1.0 cm $\times$ 45.8 cm; Gradient elution with 0.005 M Acetate buffer and 0.5 M Acetate buffer (pH 5.8).

Comparison in Mode of the Action of *Pellicularia* PDase and Snake Venom PDase towards bis-*p*-NPP. It was examined whether *Pellicularia* PDase attacked bis-*p*-NPP, generally used as the synthetic substrate for PDase activity. The preparations were partially purified enzyme, acetone fraction (P-II) and Amberlite XE-64 chromatography fractions number 53, 55 and 56. The reaction condition was described in Methods. The pH 8.5 and 5.0 were applied as incubation pH for snake venom and *Pellicularia* PDase as indicated in Table VI.

Any *Pellicularia* preparations could never act on bis-*p*-NPP at both pH values, quite unlikely to RNA, whereas snake venom PDase hydrolyzed it considerably even in small amount. Therefore, it should be noted that PDase in *Pellicularia*, which exhibits no activity on bis-*p*-NPP, is of different kind from snake venom enzyme and must be a new type PDase.

DISCUSSION

Pellicularia PDase is activated by Zn^{++} and designated to Zn^{++} -enzyme as described in

TABLE VI. COMPARISON OF THE ACTION OF *Pellicularia* PDASE AND SNAKE VENOM PDASE TOWARDS BIS-*p*-NPP

Enzyme	PDase Activity		
	pH 5.0		pH 8.5
	O.D. at 660 $m\mu$ **	O.D. at 430 $m\mu$ *	O.D. at 430 $m\mu$ *
Snake Venom	Trace	0.110	0.650
Acetone fraction P-II	0.800	0.050	0.010
Amberlite XE-64 fraction			
No. 53	0.900	0.015	0.010
55	1.110	0.020	0.007
56	1.990	0.015	0.005

* Reaction mixture: Bis-*p*-NPP, 5 mole, 1.0 ml; Enzyme, 1.0 ml; 37°C, 2 hr.

** Reaction mixture: 5% RNA, 0.2 ml; 0.143 M Veronal buffer (pH 5.0), 1.0 ml; Enzyme, 0.1 ml; H_2O , 0.7 ml; 60°C, 10 min.

Value of activity was calculated as 1.0 ml of each fraction of Amberlite XE-64.

Results. There have been found several metal enzymes, for example, Ca^{++} -binding α -amylase, Zn^{++} -binding alkaline phosphatase of

E. coli found by Vallee and his co-workers²⁷⁾ and so on. There are until now few reports which show that PDase was activated by Zn^{++} , and that PDase inactivated completely by EDTA was reversibly restored by only Zn^{++} among the metals so far tested.

In most cases it is accepted that Zn^{++} is a potent inhibitor for PDase, i.e. for snake venom PDase. PDase in leukemic cells of mouse²⁸⁾ was accelerated by Mg^{++} or Mn^{++} . Of nucleases reported recently, calf pancreas PDase activity found by Terao and Ukita²⁹⁾ was dependent on Co^{++} , that of *B. subtilis*²¹⁾ on Ca^{++} and that of *N. crassa*^{9,10)} on Ca^{++} , Mg^{++} , Fe^{++} and Na^{+} . There have been found also Mg -dependent potato nuclease I and micrococcal nuclease.³¹⁾ It could be assumed that *Pellicularia* PDase consists of activator and functional metal as shown in chart. The one was easily removed by H_2O -dialysis and reactivated by aliquot addition of Mn^{++} , Mg^{++} and Zn^{++} , but the other retained its function until being captured by EDTA, chelating agent to seize the functional Zn^{++} . Zn^{++} addition reversibly recovered the enzyme activity lost completely by EDTA pretreatment.

It is interesting that the *Pellicularia* produces also other enzymes, which can be activated by Zn^{++} like PDase, i.e. protease and 3'-nucleotidase. Two PDases were partially separated by Amberlite XE-64 chromato-

graphy. Suzuki and Iwanaga^{32,33)} demonstrated three PDases, PDase, I, II and III, from Japanese Mamushi venom.

Recently Lehman and Nussbaum¹¹⁾ and Kornberg et al.^{12,13)} proved the properties of exonuclease (PDase) I, II and III in *E. coli*. Also endonuclease I and II of *E. coli*^{34,35)} and potato tubers³⁰⁾ were found.

At the end, substrate specificity of the enzyme should be discussed. *Pellicularia* PDase is thought to be incapable of splitting bis-*p*-NPP. Leukemic cell PDase of mouse carrying ascites tumor, isolated by Anderson,²⁸⁾ as well as snake venom PDase hydrolyzes RNA, poly A, poly AU and oligonucleotides stepwise from terminal residue of the molecules. But the former cannot attack benzyladenosine 5'-phosphate or *p*-nitrophenyl thymidine 5'-phosphate.

It was shown that a Mn^{++} -dependent nuclease can digest RNA, RNase I core and DNA, but it cannot act on bis-*p*-NPP.³⁶⁾

Therefore, basing upon Zn^{++} dependency and substrate specificity, *Pellicularia* PDase must be a new type nuclease.

In order to examine the specificities of RNase associated with PDase, much more purified preparations should be made.

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