Substrate Specificity of Nuclease P_1 [†]

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Nuclease P_1 cleaved substantially all phosphodiester bonds in rRNA, tRNA, poly(I), poly(U), poly(A), poly(C), poly(G), poly(I)·poly(C), native DNA and heat-denatured DNA to produce exclusively 5'-mononucleotides. Single-stranded polynucleotides were much more susceptible than double-stranded ones. Influence of pH and ionic strength on the hydrolysis rate significantly varied with the kind of polynucleotides. The enzyme also hydrolyzed 3'-phosphomonoester bonds in 3'-AMP, 3'-GMP, 3'-UMP, 3'-CMP, 3'-dAMP, 3'-dGMP, 3'-dCMP and 3'-dTMP. Ribonucleoside 3'-monophosphates were hydrolyzed 20 to 50 times faster than the corresponding 3'-deoxyribonucleotides. Base preference of the enzyme for 3'-ribonucleotides was in the order of $G > A > C \geq U$, whereas that for 3'-deoxyribonucleotides was in the order of $C \geq T > A \geq G$. The 3'-phosphomonoester bonds in nucleoside 3', 5'-diphosphates, coenzyme A and dinucleotides bearing 3'-phosphate were hydrolyzed at a rate similar to that for the corresponding 3'-mononucleotides. Adenosine 2'-monophosphate was highly resistant, being split at less than 1/3,000 the rate at which 3'-AMP was split.

In the previous papers,^{2,3)} the evidence was presented to identify the phosphodiesterase activity toward RNA and DNA and the phosphomonoesterase activity toward 2'- and 3'-AMP with nuclease P_1 . The present investigation was undertaken to clarify the substrate specificity of nuclease P_1 . The results obtained here showed that nuclease P_1 cleaved phosphodiester bonds of polynucleotides and 2'- and 3'-phosphomonoester bonds regardless of nature of sugar and base, and that it was highly preferential for single-stranded polynucleotides and 3'-phosphomonoesters of ribonucleosides.

MATERIALS AND METHODS

Enzyme preparations. Nuclease P_1 was prepared as previously described.³⁾ Pancreatic ribonuclease A (type III), spleen phosphodiesterase (type I), snake venom phosphodiesterase (type II), micrococcal nuclease (grade V) and *E. coli* alkaline phosphatase (type III) were purchased from Sigma Chemical Co.

Substrates. Low molecular weight yeast RNA, the

mean chain length of which is about 40, was purchased from Kohjin Co., Ltd. Yeast tRNA, 3'-AMP, 3'-GMP, 3'-CMP, and 3'-UMP were purchased from Boehringer Mannheim Biochemicals Co. Calf thymus DNA, coenzyme A, 2'-AMP and 3',5'-cyclic AMP were purchased from Sigma Chemical Co. Heat-denatured DNA was prepared by heating calf thymus DNA solution (2 mg/ml), pH 7.0, at 100°C for 15 min, and then cooling it rapidly in ice water. Yeast rRNA was isolated by gel-filtration on a Sephadex G-200 column of phenol treated⁴⁾ ribonucleoprotein⁵⁾ obtained from baker's yeast. Poly (I), poly (A), poly (C), poly (U) and poly (G) were prepared in this laboratory.⁶⁾ Guanosine 3'(2'), 5'-diphosphate and thymidine 3',5'-diphosphate were prepared by the method of Ueda and Kawai⁷⁾ and Dekker *et al.*,⁸⁾ respectively. Deoxyribonucleoside 3'-monophosphates were prepared as follows: 40 ml of heat-denatured calf thymus DNA solution (5 mg/ml) pH 8.0, was incubated with 10 mg of micrococcal nuclease and 80 μ moles of $CaCl_2$ for 4 hr at 37°C. The digest was subjected to chromatography on a DEAE-Sephadex A-25 column (2 $\times$ 40 cm) with a linear gradient of sodium chloride (0.14 M ~ 0.28 M) in 0.02 M Tris-HCl and 7 M urea, pH 7.5 (total volume, 2 liters). The mononucleotide fraction was desalted by activated charcoal, and then chromatographed on a Dowex 1- $\times$ 8 column (1.5 $\times$ 20 cm) with a linear gradient of sodium formate buffer (0 ~ 0.7 M), pH 3.0, in a total volume of 1 liter. The isolated fractions of 3'-dCMP, 3'-dAMP, 3'-dTMP and 3'-dGMP were desalted by activated charcoal and then lyophilized.

Dinucleotides bearing 3'-phosphate were prepared as

[†] Studies on a Nuclease from *Penicillium citrinum*. Part III. Part II, see reference 2). A part of this paper was presented at the 4th International Fermentation Symposium, Kyoto, March, 1972.¹⁾

follows: 5 ml of heat-denatured DNA solution (4 mg/ml), pH 8.0, was hydrolyzed with 1 mg of micrococcal nuclease and 10 μ moles of CaCl_2 for 2 hr at 37°C. Two ml of RNA solution (20 mg/ml), pH 7.0, was incubated with 5 mg of pancreatic ribonuclease A for 3 hr at 37°C. Each digest was subjected to chromatography on a DEAE-Sephadex A-25 urea column (2 $\times$ 40 cm) as mentioned above. The dinucleotide fraction was desalted by gel filtration on a Sephadex G-10 column, and then each constituent dinucleotide was isolated on a paper by a two-dimensional development; electrophoresis followed by chromatography with solvent I or solvent III. As the 8 dinucleotides indicated in Table I were easily obtained singly, these compounds were

ester bonds released, 10 μ l of *E. coli* alkaline phosphatase (500 μ g/ml) was added to the reaction mixture and incubation was carried out at 37°C for 30 min. Inorganic orthophosphate liberated was determined by Allen's method¹⁰⁾ with a slight modification.

Paper electrophoresis and paper chromatography. Paper electrophoresis was performed in 3% acetic acid (pH 2.5) at about 0.4 mA/cm and 1,000 V/31 cm for 2 hr. The following solvent systems were used for descending paper chromatography: Solvent I, isobutyric acid—28% ammonia water—water (50: 1: 29); solvent II, saturated aqueous ammonium sulfate—isopropanol—1 M sodium acetate (40: 1: 9); solvent III, *n*-butanol—

TABLE I. DINUCLEOTIDES SEPARATED FROM PANCREATIC RIBONUCLEASE A DIGEST OF RNA AND FROM MICROCOCCAL NUCLEASE DIGEST OF HEAT-DENATURED DNA

Compound ^{a)}	Origin	Phosphodiesterase hydrolysis of dephosphorylated product ^{b)}		Corresponding dinucleotide
		Venom	Spleen	
1	RNA digest	A + pC	Ap + C	A - Cp
2	"	G + pC	Gp + C	G - Cp
3	"	A + pU	Ap + U	A - Up
4	"	G + pU	Gp + U	G - Up
1	DNA digest	dT + dpT	dTp + dT	d(T - Tp)
2	"	dT + dpA	dTp + dA	d(T - Ap)
3	"	dA + dpT	dAp + dT	d(A - Tp)
4	"	dT + dpG	dTp + dG	d(T - Gp)

^{a)} Order of electrophoretic mobility toward anodeside: G-Up > A-Up > G-Cp > A-Cp; d(T-Tp) > d(T-Gp) $\geq$ d(T-Ap) $\approx$ d(A-Tp). *Rf* values in solvent I: G-Up, 0.12; G-Cp, 0.27; A-Up, 0.40; A-Cp, 0.49; d(T-Gp), 0.25; d(T-Tp), 0.30; d(T-Ap), 0.42; d(A-Tp), 0.46.

^{b)} Each compound was first dephosphorylated with *E. coli* alkaline phosphatase and, after electrophoretic purification, further treated with excess of venom and spleen phosphodiesterases separately. The resultant products were chromatographically and spectrophotometrically determined.

characterized and employed as substrates for nuclease P_i . Ribose-3-phosphate was prepared from 3'-XMP by acid hydrolysis according to Leven's method⁹⁾ and deoxyribose-3-phosphate was prepared from 3'-dAMP by the same method.

Assay of enzyme activity. Phosphomonoesterase activity toward various substrates was assayed by measuring the amount of inorganic phosphate liberated during incubation by the standard method as described in the previous paper³⁾ except that various buffers were used. The phosphodiesterase activity was assayed by measuring the amount of phosphomonoester bonds liberated from polynucleotides during incubation. The reaction mixture containing 0.2 ml of a buffer, 0.2 ml of polynucleotides solution (5 mg/ml) and 0.1 ml of the enzyme solution was incubated at 37°C for 15 min. The reaction was stopped by adding 0.5 ml of 0.2 M Tris-HCl buffer, pH 9.1. To hydrolyze phosphomono-

acetic acid—water (4: 3: 2).

Identification of the digestion products of polynucleotides. The digestion products of polynucleotides were characterized by paper electrophoresis, paper chromatography with solvent I, II or III and spectrophotometry.

RESULTS

Specificity for polynucleotides

In Fig. 1 pH-activity curves toward various kinds of polynucleotides are shown. The influence of pH on the rate of hydrolysis varied with the kind of polynucleotides. The marked difference of pH dependence was observed among homopolymers. Poly(A) and poly(C) were rapidly attacked at pH around 6, but were

FIG. 1. Effect of pH on Phosphodiesterase Activity toward Various Polynucleotides.

Enzyme activity toward each substrate was assayed by the standard method except that the following buffers were used: M/20 acetate buffer, pH 3.1~4.5; M/35 veronal buffers, pH 4.5~6.5; M/20 Tris-HCl buffer, pH 6.5~7.0.

A: Activity toward naturally occurring nucleic acids ○—○, low molecular weight RNA; ■—■, tRNA; ●—●, rRNA; □—□, heat-denatured DNA.

B: Activity toward synthetic homopolynucleotides △—△, poly (A); ▲—▲, poly (I); □—□ poly (C); ■—■, poly (U).

highly resistant at pH 4.5. On the other hand, poly(U) and poly(I) were attacked rapidly in the range from pH 4 to 5, but very slowly at

pH around 6.0. The difference in pH dependence could be seen between low molecular weight RNA and high molecular weight one, too. Although these results are difficult to understand systematically, it appears that the difference in pH dependence of the reaction can be caused from that of the conformation of the substrates.

Effect of ionic strength on susceptibility of various polynucleotides was also examined (Fig. 2). An increase in NaCl concentration was generally found to reduce the rate of hydrolysis and to shift the pH optimum to acidic side. In the presence of 0.2 M NaCl poly(U) and poly(C) were highly resistant, whereas under the identical condition poly(A) was susceptible. The effect of ionic strength on the reaction rate also appears to reflect conformational change of the substrate.

The hydrolysis rates for various polynucleotides at the optimal pH are given in Table II. Single-stranded polynucleotides were much more rapidly attacked than double-stranded polynucleotides. The rate of hydrolysis decreased in the order of poly(I) > poly(A) > poly(U) > low molecular weight RNA ≥ poly(C) > tRNA ≥ rRNA > heat-denatured DNA ≫ poly (I) · poly (C) ≥ poly (G) ≫ native DNA. The *K_m* values for RNA and heat-

FIG. 2. Effect of NaCl on Phosphodiesterase Activity toward Synthetic Homopolymers.

Enzyme activity toward each substrate was assayed as described in the legend to Fig. 1 except that sodium chloride was added to the reaction mixture.

○—○, none; ●—●, 0.1 M NaCl; □—□, 0.2 M NaCl; ■—■, 0.4 M NaCl.

TABLE II. PHOSPHODIESTERASE ACTIVITY TOWARD VARIOUS POLYNUCLEOTIDES AT THE OPTIMAL pH

Enzyme activity toward each substrate was assayed as described in the legend to Fig. 1.

	Opt. pH	Hydrolysis rate ^{a)}
Low molecular weight RNA	6.0	336.0
tRNA	5.3	262.1
rRNA	5.3	245.3
Heat-denatured DNA	5.3	218.4
Native DNA	5.3	1.1
Poly (A)	6.0	490.1
Poly (I)	4.5	541.0
Poly (C)	6.0	305.8
Poly (U)	4.0	390.0
Poly (G)	4.5	14.0
Poly (I)·poly (C)	4.8	16.8

^{a)} μ moles of phosphodiester cleaved per 1 mg of the enzyme in 1 min at 37°C.

denatured DNA at pH 5.3 were calculated from a double reciprocal plot to be 5×10^{-5} M and 3×10^{-5} M (on P basis), respectively.

If oligonucleotides had been present in the digests, most of them should have migrated between UMP and GMP in paper electrophoresis. Such oligonucleotides, however, were not detected in the nuclease P_1 digests of rRNA, DNA and homopolynucleotides after exhaustive digestion. Such oligonucleotides were not detected in the digest of tRNA either, although appreciable amounts of ϕ UMP and several methylated nucleotides were recognized. Electrophoretic mobility and R_f value in solvent I of ϕ UMP were a little smaller than those of UMP. On the other hand, electrophoretic mobilities of the methylated nucleotides detected were essentially the same as those of the corresponding major mononucleotides, while R_f values in solvent I of the methylated nucleotides were a little greater than those of major mononucleotides. It may be safe to conclude, therefore, that nuclease P_1 completely hydrolyzed normal 3'-5' phosphodiester linkages in RNA and DNA, irrespective of basestructure, giving rise to 5'-mononucleotides.

Specificity for phosphomonoesters

1) Action on 3'-nucleotides. In Fig. 3

FIG. 3. Effect of pH on Phosphomonoesterase Activity for Various Substrates.

The enzyme activity toward each substrate was assayed by the standard method except that the following buffers were used: M/20 acetate buffer, pH 3.1~pH 4.5; M/35 veronal buffer, pH 4.5~6.5; M/20 Tris-HCl buffer, pH 6.5~9.5.

A: Activity toward 3'-ribonucleotides ○—○, 3'-AMP; ●—●, 3'-GMP; □—□, 3'-CMP; ■—■, 3'-UMP
B: Activity toward 3'-deoxyribonucleotides ○—○, 3'-dAMP; ■—■, 3'-dCMP; □—□, 3'-dTMP.

pH-activity curves toward various kinds of 3'-nucleotides are shown. The influence of pH on the rate of hydrolysis varied with the kind of nucleotides. It is noted that the pH optima for 3'-deoxyribonucleotides were lower than those for the corresponding 3'-ribonucleotides. The reaction rates for various 3'-nucleotides at the optimal pH are shown in Table III. Hydrolysis of 3'-ribonucleotides was much faster than that of 3'-deoxyribonucleotides, and base preference of nuclease P_1 for 3'-ribonucleotides was different from that for 3'-deoxyribonucleotides: The reaction rate in 3'-ribonucleotide decreased in the order of 3'-GMP > 3'-AMP > 3'-CMP $\geq$ 3'-UMP, whereas in 3'-deoxyribonucleotide the rate decreased in the order of 3'-dCMP $\geq$ 3'-dTMP > 3'-dAMP $\geq$ 3'-dGMP. K_m and V_{max} values with various 3'-nucleotides were determined from the Lineweaver-Burk plots (Table IV). In the case of 3'-deoxyribonucleotides V_{max} values were very low relative to their K_m values as compared with 3'-ribonucleotides.

Even with 10,000 times the amount of enzyme required to hydrolyze 3'-AMP, no hydro-

TABLE III. PHOSPHOMONOESTERASE ACTIVITY TOWARD VARIOUS NUCLEOTIDES AT THE OPTIMAL pH

Enzyme activity toward each substrate was assayed as described in the legend to Fig. 3.

	Opt. pH	Hydrolysis rate ^{a)}
3'-AMP	7.2	1,004.3
3'-GMP	8.5	1,440.0
3'-CMP	6.0	853.6
3'-UMP	6.0	700.3
3'-dAMP	4.5	16.7
3'-dGMP	5.0	14.0
3'-dCMP	4.5	49.6
3'-dTMP	4.5	33.6
pGp	8.5	1,296.0
dpTp	4.5	18.1
Coenzyme A	6.0	662.9
2'-AMP	6.0	0.3

^{a)} μ moles of P_i formed per 1 mg of the enzyme in 1 min at 37°C.

TABLE IV. *K_m* AND *V_{max}* FOR VARIOUS 3'-NUCLEOTIDES

A reaction mixture containing 0.5 ml of enzyme solution, 1 ml of substrate and 1 ml of M/35 veronal buffer, pH 5.3, was incubated at 37°C for 15 min. After incubation inorganic phosphate liberated was determined.

Substrates	<i>K_m</i>	<i>V_{max}</i> (μ moles/mg enzyme/min)
3'-AMP	7.0×10^{-5} M	341.4
3'-GMP	9.0×10^{-5} M	290.0
3'-CMP	1.6×10^{-4} M	462.2
3'-UMP	2.5×10^{-4} M	435.5
3'-dAMP	1.6×10^{-4} M	10.5
3'-dCMP	4.0×10^{-4} M	38.2
3'-dTMP	6.0×10^{-4} M	29.4

lysis was detected with ribose-3-phosphate, deoxyribose-3-phosphate, 5'-ribonucleotides, 5'-deoxyribonucleotides, ADP, ATP, 3', 5'-cyclic AMP and *p*-nitrophenylphosphate.

2) *Action on dinucleotides bearing 3'-phosphate.* The action of nuclease P₁ toward various dinucleotides bearing 3'-phosphate was also examined to see whether the 3'-terminal phosphate of nucleic acids can be attacked or not. As one of examples the action toward A-Up is illustrated in Table V. Although the

TABLE V. TIME-COURSE OF DIGESTION OF A-Up

A 0.05 ml reaction mixture (pH 5.0) containing 3 A₂₆₀ units of A-Up and 0.01 μ g of nuclease P₁ was incubated at 37°C. At the indicated reaction time, the digestion products were separated by the combination of electrophoresis and chromatography on a paper and subjected to spectrophotometry.

Incubation time (min)	A ₂₆₀ unit in 0.1 N HCl				
	A-Up	Ado	A-U	pU	pUp
0	3.00	0	0	0	0
5	2.08	0.10	0.62	0	0.06
30	0.40	1.06	1.22	0.40	0.15
150	0	1.85	0	1.27	0

R_f values of pUp, pU, A-Up, A-U and Ado in solvent I were 0.17, 0.22, 0.38, 0.50 and 0.73, respectively.

Order of electrophoretic mobility toward anode side was pUp $\gg$ pU > A-Up > A-U > Ado.

substrate was finally hydrolyzed to adenosine and 5'-UMP with liberation of 3'-terminal phosphate, A-U and pUp were observed to accumulate as intermediates at the early stage of incubation. This result indicates that nuclease P₁ attacks A-Up in 2 ways: (1) A-Up $\rightarrow$ A-U + P_i; A-U $\rightarrow$ Ado + pU. (2) A-Up $\rightarrow$ Ado + pUp; pUp $\rightarrow$ pU + P_i. The reaction (1) appears to occur predominantly, because A-U was produced much faster than any other products Ado, pU or pUp. Other dinucleotides bearing 3'-phosphate, A-Cp, G-Up, G-Cp, d(T-Ap), d(T-Tp) and d(G-Tp) were also confirmed to be attacked by the same mode of action as A-Up.

DISCUSSION

From the results described above, it is now clear that nuclease P₁ cleaved substantially all phosphodiester bonds in rRNA, tRNA, DNA and synthetic homopolymers to produce exclusively 5'-mononucleotides. Therefore it would appear that the nuclease P₁ is totally blind to the nature of sugar and base, at least in the qualitative sense. In the quantitative sense, however, the enzyme seemed to have a unique preference with regard to substrate conformation. The unusual effect of pH or ionic strength on the susceptibility of synthetic homopolymers seems to reflect the importance

of substrate conformation for catalysis. Nuclease P_1 had a high degree of preference toward single-stranded polynucleotides. Therefore, low concentration of nuclease P_1 would be able to remove single-stranded DNA exclusively from a mixture of single-stranded and double-stranded DNA. In this respect, nuclease P_1 is similar to nuclease S_1 ¹¹⁾ and nuclease O ¹²⁾ from *Aspergillus oryzae* and a nuclease from *Neurospora crassa*.¹³⁾

In addition, nuclease P_1 complexed with malonogalactan¹⁴⁾ degraded double-stranded DNA at much lower rate than the polysaccharide-free enzyme. This point will be discussed in detail in the following paper of this series.

Nuclease P_1 was able to hydrolyze 3'-phosphomonoester bonds of various kinds of 3'-mononucleotides, nucleoside 3', 5'-diphosphates, coenzyme A and oligonucleotides bearing 3'-terminal phosphate. Therefore, the phosphomonoesterase activity of the enzyme is also concluded to be unspecific with the nature of sugar, base, and substituent on the 5'-position, although the type of sugar was observed to affect the affinity and the maximal rate of hydrolysis much more greatly than the nature of base or substituent on the 5'-position does. The hydroxyl group on the 2'-position seems to be important for the enzyme action. The base moiety must be an essential feature of the substrate because ribose-3-phosphate or deoxyribose-3-phosphate was not split by nuclease P_1 .

To nuclease P_1 2'-AMP was fairly resistant, being split at less than 1/3,000 the rate at which 3'-AMP was split. The hydrolysis proceeded linearly with time, and the rate depended upon the amount of enzyme. In addition, 2'-AMP preparation employed had previously been confirmed to be free of 3'-AMP. From the data now available, it is conceivable that the activity toward 2'-nucleotide is not due to any contaminating enzyme, but due to an intrinsic catalytic property of nuclease P_1 itself. Nuclease P_1 is a useful tool for preparing 2'-nucleotides from alkaline hydrolysate of RNA because 3'-nucleotides would be exclusively

broken down by a low level of the enzyme.

Substrate specificity of nuclease P_1 is similar to that of other analogous bifunctional enzymes from *Phoma cucurbitacearum*,¹⁶⁾ mung beans,¹⁶⁾ *Monascus purpureus*¹⁷⁾ and potato¹⁸⁾ in the following points: (1) They are 5'-monoester-forming nucleases, non-specific toward sugar and base. (2) They are highly preferential for single-stranded structure. (3) They show high phosphomonoesterase activity toward 3'-nucleotides, but 2'-nucleotides are extremely resistant to them. However, they are different from one another in their pH optima and relative reaction rates for various substrates. Among them only mung bean enzyme, as well as nuclease P_1 , was reported to be able to split 3'-terminal phosphomonoester linkage in polynucleotide. It is interesting to note that mung bean nuclease I was able to cleave the internucleotide bond in dinucleotide bearing 3'-phosphate only after removal of 3'-terminal phosphate, whereas nuclease P_1 could cleave the internucleotide bond both before and after removal of 3'-terminal phosphate.

Nuclease P_1 is expected to be a useful reagent for analysis of base composition of RNA and DNA because it can split substantially all phosphodiester bonds in nucleic acids regardless of nature of sugar and base. Mode of action of nuclease P_1 will be described in the subsequent paper of this series.

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