Degradation of Nucleic Acids and their Related Compounds by Microbial Enzymes

Part III. On the Distribution of Extracellular Enzymes Capable of Degrading Deoxyribonucleic Acid into 5'-Deoxymononucleotides in Microorganisms.*

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Received October 22, 1962

The distribution in microorganisms of extracellular enzymes which degrade DNA into deoxymononucleotides was studied. The degradation products of DNA were determined by using 5'-nucleotidase and prostatic nonspecific phosphomonoesterase. It was found that except *Bacillus subtilis* IFO 3302, the microorganisms which produced the enzymes that catalyze the degradation of RNA into 5'-mononucleotides, produced the enzymes capable of hydrolyzing DNA into 5'-deoxymononucleotides, whereas the microorganisms which produced such enzymes that degrade RNA into 3'-mononucleotides did not generally produce the enzymes which hydrolyze DNA.

INTRODUCTION

It was previously reported that the phosphodiesterases capable of hydrolyzing RNA into 5'-mononucleotides were produced by various microorganisms¹⁾, and other microorganisms produced the phosphodiesterases capable of degrading RNA into 3'-mononucleotides²⁾. From these findings, it has become possible to produce economically 5'-mononucleotides and 3'-mononucleotides, the former is regarded as a new chemical seasoning agent. Deoxymononucleotides and their derivatives are important not only as biochemical reagents but also as medicines. However, it is difficult to produce deoxymononucleotides on a large scale. In order to

produce them economically, we searched for species and strains which produce extracellular enzymes capable of degrading DNA into deoxymononucleotides. The present communication is concerned with the distribution of the enzymes in microorganisms.

EXPERIMENTAL

Materials.

Organisms. All the strains were supplied by the Institute for Fermentation, Osaka.

Enzyme solutions were prepared by the same method as that described in the previous paper²⁾.

Enzymes for Assay. 5'-Nucleotidase and non-specific phosphomonoesterase were prepared by the methods previously described²⁾. Sperm DNA and calf thymus DNA were purchased from Nutritional Biochemicals Corporation and Worthington Biochemical Corporation, respectively.

Methods. In order to search for the strains, which produce enzymes capable of degrading DNA into deoxymononucleotides, a large number of microorganisms were subjected to screening tests consisting of two steps.

* The report was presented at the Meeting of the Kanto Division of the Agricultural Chemical Society of Japan, Tokyo, November 12, 1960.

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The abbreviations used are: RNA, Ribonucleic acid; DNA, Deoxyribonucleic acid; DNase, Deoxyribonuclease; TCA, Trichloroacetic acid; Tris buffer, Tris (hydroxymethyl) aminomethane buffer.

1) K. Ogata, et al., This journal, **27**, 110 (1963).

2) Y. Nakao and K. Ogata, *ibid.*, **27**, 116 (1963).

In the first screening test, the activities of DNase and phosphomonoesterase in the enzyme preparations were examined. In the second screening test, it was tested whether the enzyme preparations could degrade DNA into 5'-deoxymononucleotides or 3'-deoxymononucleotides.

The First Screening Test. Although many methods³⁾ of assaying DNase have been reported, McDonald colorimetric procedure⁴⁾ is probably the most convenient for routine measurements of DNase in many enzyme preparations. DNase activity was measured, therefore, by the method of McDonald with some modification.

One-half ml of 1.0% sperm DNA solution, 1.0 ml of 0.5 M acetate buffer (pH 5.0) or 0.5 M Tris buffer (pH 7.5) and 0.5 ml of an enzyme solution were mixed. After incubation at 37°C for 120 minutes, the reaction was stopped by addition of 2.0 ml of a modified McFadyen's reagent⁵⁾ (UPCA solution, 0.25% uranyl acetate in 2.5% perchloric acid). The mixture was left in an ice-water bath for 15 minutes, then the precipitate was removed by centrifugation. One-half ml of the supernatant was diluted to 2.0 ml with distilled water and then mixed with 4.0 ml of the diphenylamine reagent⁶⁾. The mixture was heated for 10 minutes in a boiling water bath, and after cooling, the optical density (D) at 595 m μ was read in a Hitachi EPU-2A spectrophotometer with a 1 cm light path against the control. Controls were run in each experiment by addition of the substrate after the addition of UPCA solution. The rate of DNA-degradation was calculated from the following equation;

$$D/T \times 100$$

D: the optical density at 595 m μ measured as above.

T: the optical density at 595 m μ measured by the following method. One-half ml of 1.0% sperm DNA solution was diluted to 4.0 ml with distilled water and 0.5 ml aliquot was treated by the same method as described above and then the optical density (T) at 595 m μ was measured.

The orthophosphate liberated during the degradation of DNA was determined by the method of

Fiske-Subbarow⁷⁾ as follows: four tenths ml of the supernatant fluid, 0.2 ml of 10 N H₂SO₄, 0.4 ml of 2.5% ammonium molybdate and 0.2 ml of reducing reagent were mixed and diluted to 5.0 ml with distilled water, and was left to stand for 20 minutes at 37°C; the optical density (Pi) of the mixture at 660 m μ was read in a Coleman Junior spectrophotometer against the control. The rate of orthophosphate liberation was calculated from the following equation:

$$Pi/T \times 100.$$

Pi: the optical density at 660 m μ measured as above.

T: the optical density at 660 m μ measured by the following method. One-half ml of 1.0% sperm DNA solution was mixed with 1.6 ml of 10 N H₂SO₄ and DNA-P was converted to orthophosphate by heating the mixture. The mixture was cooled, and adjusted to 10 ml with distilled water, and the orthophosphate in 1.0 ml aliquot of the solution was determined as described above without further addition of sulfuric acid.

The Second Screening Test. This test was carried out to estimate the degradation products of DNA. In this case, sodium arsenate was added to the reaction mixture to inhibit phosphomonoesterase activity, and the rates of DNA degradation and of formation of 5'- and 3'-deoxymononucleotides were estimated. The procedure is described below.

The total volume of an incubation mixture of 2.0 ml was made to contain 0.5 ml of 1.0% sperm DNA solution, 0.2 ml of 0.25 M sodium arsenate, 0.3 ml of 1 M acetate buffer (pH 5.0) or 1 M Tris buffer (pH 7.5) and an enzyme solution. The amount of the enzyme solution necessary for complete hydrolysis of DNA was determined from the results of the first screening test. After the mixture was incubated for 8 hours at 37°C, a 1.0 ml aliquot mixed with 1.0 ml of UPCA solution, and the rates of DNA degradation and of orthophosphate liberation were determined by the method described in the first screening test.

Another 1.0 ml aliquot was diluted to 5.0 ml with distilled water and heated in a boiling water bath for 5 minutes to stop the reaction. This solution was used as a sample for the determination of 5'- and 3'-deoxymononucleotides. The 5'- and 3'-deoxymononucleotides in 0.4 ml portions of the sample were respectively determined by the method described in the previous paper²⁾. Rate of the formation of 5'- or 3'-deoxymononucleotides was represented respectively in percentage of 5'-deoxymononucleotide phosphate or

3) N.B. Kurnick, "Methods of Biochemical Analysis", Vol. 9, Interscience Publishers, 1962, p. 1.

4) S.P. Colowick and N.O. Kaplan, "Methods in Enzymology", Vol. II, Academic Press, 1955, p. 437.

5) S.P. Colowick and N.O. Kaplan, *ibid.*, Vol. III, Academic Press, 1955, p. 771.

6) E. Chargaff and J.N. Davidson, "The Nucleic Acids", Vol. I, Academic Press, 1955, p. 287.

7) C.H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

3'-deoxymononucleotide phosphate in the enzymatic hydrolyzate to total phosphates in the DNA used as substrate.

RESULTS

I. Distribution of Enzymes Capable of Hydrolyzing DNA into Deoxymononucleotides in *Streptomyces* and Bacteria.

Table I illustrates the result obtained. DNA-depolymerases in the culture media of *Streptomyces* and bacteria were active at the alkaline pH and hydrolyzed DNA into 5'-deoxymononucleotides.

II. Distribution of Enzymes Capable of Degrading DNA into Deoxymononucleotides in Molds.

The results are shown in Table II. About 90 strains were tested and DNA-depolymerases in the culture media of molds were generally active at the alkaline pH, while the strains

belonging to *Monascus* and *Phytophthora infestans* produced enzymes which were active at the acid pH. However, all the enzymes degraded DNA into 5'-deoxymononucleotides.

Furthermore we tested about 50 strains of yeasts for their ability to produce DNA-depolymerase but no strain capable of hydrolyzing DNA was found.

In the preliminary experiments, we found that sperm DNA employed as substrate in these studies was partially degraded during the extraction procedures because it was in part soluble in 3 M TCA solution. So we tested DNA-depolymerase activity of the enzyme preparations using thymus highly polymerized DNA as substrate. As a result, it was found that the enzymes which was produced by the strains described in Tables I and II

TABLE I. DISTRIBUTION OF ENZYMES CAPABLE OF HYDROLYZING DNA INTO DEOXYMONONUCLEOTIDES IN BACTERIA AND *Streptomyces*

IFO No.	Microorganism	First screening				Second screening				
		Degradation of DNA (%)		Liberation of Pi* (%)		pH of Degradation	Degradation of DNA (%)	Formation of 5'-nucleotide (%)	Formation of 3'-nucleotide (%)	Liberation of Pi* (%)
		pH 5.0	pH 7.5	pH 5.0	pH 5.0					
3022	<i>Bacillus subtilis</i>	0	0	0	0					
3032	" "	0	0	0	0					
3010	" "	0	21.5	0	7.6	7.5	56.0	24.5	0	28.5
3331	" <i>brevis</i>	0	40.0	0	23.5	"	74.3	42.0	0	21.5
3028	" <i>pumilus</i>	0	0	0	0					
3132	" <i>cereus</i>	0	0	0	0					
3366	<i>Escherichia coli</i>	0	0	0	0					
3140	<i>Pseudomonas riboflavina</i>	0	0	0	0					
3080	" <i>aeruginosa</i>	0	0	0	0					
3062	<i>Micrococcus subflavus</i>	0	0	0	0					
3242	" <i>flavus</i>	0	0	0	0					
3333	" <i>lysodeikticus</i>	0	0	0	0					
3060	" <i>pyogenes</i> var. <i>aureus</i>	0	0	0	0					
3183	" "	0	0	0	0					
3332	" <i>citreus</i>	0	0	0	0					
3380	<i>Erwinia carotovora</i>	0	15.4	0	5.9	7.5	52.0	20.1	0	23.1
3303	<i>Streptomyces aureus</i>	8.0	73.0	3.0	50.0	"	100.0	52.0	0	31.5
3113	" <i>viridochromogenus</i>	0	13.8	0	0	"	71.0	26.0	0	0
3389	" <i>purpurascens</i>	0	20.0	0	5.4	"	55.0	21.5	0	7.5
	" <i>spec.</i>	0	36.5	0	27.0	"	100.0	26.0	0	10.5

*Pi: orthophosphate.

TABLE II. DISTRIBUTION OF ENZYMES CAPABLE OF HYDROLYZING DNA INTO DEOXYMONO-NUCLEOTIDES IN MOLDS

IFO No.	Microorganism	First screening				Second screening				
		Degradation of DNA (%)		Liberation of Pi* (%)		pH of Degradation	Degradation of DNA (%)	Formation of 5'-nucleotide (%)	Formation of 3'-nucleotide (%)	Liberation of Pi* (%)
		pH 5.0	pH 7.5	pH 5.0	pH 7.5					
4434	<i>Absidia butleri</i>	0	0	0	0					
4435	" <i>coerulea</i>	0	0	0	0					
5873	" <i>spinosa</i>	0	6.0	0	0					
5242	<i>Acrocyllidium spec.</i>	20.0	90.8	14.0	49.5	7.5	96.0	43.0	0	21.5
5864	<i>Aspergillus fonsecaeus</i>	0	15.4	0	0	"	40.1	40.0	0	0
4031	" <i>foetidus</i>	0	12.0	0	0	"	51.5	34.0	0	0
4053	" <i>flavus</i>	0	15.4	0	0	"	56.5	36.5	0	0
4066	" <i>niger</i>	0	0	0	0					
5374	" "	0	0	0	0					
5376	" "	0	0	0	0					
5866	" <i>fischeri</i>	0	34.0	0	5.4	7.5	90.0	68.0	0	2.7
4052	" <i>flavipes</i>	0	18.4	0	0	"	100.0	74.0	0	2.7
4339	" <i>melleus</i>	0	17.0	0	3.8	"	95.0	71.0	0	0
4363	" <i>quercinus</i>	0	38.5	0	2.0	"	82.0	67.0	0	0
4094	" <i>sulphureus</i>	0	51.0	0	3.8	"	70.7	63.0	0	0
5483	<i>Anixiella reticulispora</i>	0	15.2	0	2.1	"	86.0	58.0	0	14.0
5991	<i>Blackeslea circinans</i>	0	0	0	0					
4837	<i>Botryoshaeria ribis chromogena</i>	0	40.0	0	5.3	7.5	95.0	66.0	3.0	8.0
6142	<i>Corticium centrifugum</i>	6.2	10.7	5.9	0.5					
4442	<i>Cunninghamella echinulata</i>	6.2	10.7	3.5	2.7	7.5	27.5	10.5	0	0
4441	" <i>elegans</i>	0	7.7	0	1.0	"	25.5	10.0	0	0
4451	<i>Chaetomidium japonicum</i>	0	16.0	0	8.0	"	67.6	37.0	0	31.0
5421	<i>Fusarium roseum</i>	4.0	10.6	0	0	"	71.4	59.5	0	8.6
5893	" <i>solani</i>	0	15.4	0	5.4	"	74.3	47.5	0	21.5
5952	<i>Gloeosporium laeticolor</i>	0	10.8	0	0	"	80.0	76.0	0	0
5907	<i>Glomerella cingulata</i>	6.0	37.0	0	4.3	7.5	91.0	72.0	0	5.4
4871	<i>Gliomastix convoluta</i>	0	37.0	0	14.2	"	80.0	44.5	0	28.5
4475	<i>Helicostylum piriforme</i>	0	0	0	0					
5273	<i>Helminthosporium sigmoideum</i> var. <i>irregulare</i>	5.0	63.0	2.0	20.0	7.5	92.4	60.0	0	11.8
5367	<i>Irpex lacteus</i>	0	0	0	0					
4946	<i>Lenzites tenuis</i>	0	0	0	0					
4480	<i>Monascus pilosus</i>	50.8	29.2	31.2	10.2	5.0	35.5	20.0	0	2.0
4485	" <i>major</i>	94.0	64.7	76.3	25.8	5.0	24.5	18.5	0	2.0
4487	" <i>serorubescens</i>	58.5	30.8	35.5	3.8	5.0	44.6	38.0	0	3.0
4489	" <i>albidus</i>	61.5	47.7	32.5	5.4	5.0	55.5	33.5	0	4.8
4498	" <i>spec.</i>	33.3	50.8	11.8	5.4	7.5	54.0	39.0	0	2.0
4478	" <i>anka</i>	10.0	24.5	5.4	2.7	7.5	38.5	28.0	0	0
4578	<i>Mucor pusillus</i>	6.2	4.6	4.3	1.6					
4585	" <i>genevensis</i>	0	5.0	0	0					
6067	<i>Neurospora crassa</i>	3.0	21.5	2.0	7.8	7.5	87.7	58.0	0	22.0
6069	" <i>sitophila</i>	14.0	37.0	8.5	9.2	"	96.0	50.0	0	28.5
6026	<i>Penicillium citrinum</i>	4.6	0	2.1	0					
6092	" <i>corymbiferum</i>	3.1	1.5	2.7	0					

(Continued)

IFO No.	Microorganism	First screening				Second screening				
		Degradation of DNA (%)		Liberation of Pi* (%)		pH of Degradation	Degradation of DNA (%)	Formation of 5'-nucleotide (%)	Formation of 5'-nucleotide (%)	Liberation of Pi* (%)
		pH 5.0	pH 7.5	pH 5.0	pH					
6090	<i>Penicillium baarnense</i> var. <i>beyma</i>	6.2	3.1	5.0	0					
6088	" <i>asperum</i>	6.0	0	3.4	0					
6014	" <i>crustosum</i>	7.7	29.2	0	4.3	7.5	37.0	32.5	0	0
5282	<i>Pestalotia diospyri</i>	10.8	24.6	10.7	1.0	"	74.0	56.0	0	15.6
5427	<i>Pestalotiopsis funerea</i>	7.7	29.2	7.5	3.2	"	51.0	35.0	0	10.0
5822	<i>Phycomyces blakesleeana</i>	0	0	0						
4872	<i>Phytophthora infestans</i>	86.0	47.7	55.5	9.7	5.0	49.5	31.0	0	4.8
4935	<i>Polyporus orientalis</i>	0	0	0	0					
4917	<i>Polystictus hirsutus</i>	0	0	0	0					
4937	" <i>versicolor</i>	0	0	0	0					
4927	<i>Poria vaporaria</i>	0	0	0	0					
5911	<i>Sclerotinia fuckeliana</i>	0	0	0	0					
4745	<i>Rhizopus chinensis</i>	0	0	0	0					
4759	" <i>niveus</i>	0	0	0	0					
5696	<i>Tilachlidium humicola</i>	4.0	89.0	2.0	27.0	7.5	100.0	50.0	0	28.5

*Pi: orthophosphate.

hydrolyzed the thymus highly polymerized DNA also.

DISCUSSION

It is apparent from the data presented here that large number of microorganisms such as *Streptomyces*, bacteria, and molds produce extracellular enzymes which degrade DNA into 5'-deoxymononucleotides.

5'-Deoxymononucleotides have been hitherto produced from DNA by using DNase I and phosphodiesterase from snake venom⁸⁾ or intestinal mucosa⁹⁾. However, these methods possess the defect that the enzymes are not obtained in a large quantity. On the basis of the results obtained here, 5'-deoxymononucleotides may be produced easily from DNA by means of microbial enzymes. This method is very favorable for the production of 5'-deoxymononucleotides, because it is extremely easy to obtain enzyme solution on a large scale.

Previously, we investigated the distribution of RNA-hydrolyzing enzymes in various microorganisms. Comparing the present results with those in the previous reports^{1,2)}, the authors found that most of the organisms, which produce enzymes capable of degrading RNA into 5'-mononucleotides, produce the enzymes which catalyze the degradation of DNA, yielding, among other products, 5'-deoxymononucleotides.

On the other hand, the enzymes, which are produced in the culture media by various microorganisms, such as *Rhodotorula glutinis* or *Sporoboromyces pararoseus* and which degrade RNA into 3'-mononucleotides, do not hydrolyze DNA. Koerner¹⁰⁾ reported that the splenic phosphodiesterase do not hydrolyze highly polymerized DNA but degrade a splenic DNase II digest, that is polydeoxyribonucleotides having 3'-phosphomonoester end groups, to 3'-deoxymononucleotides. Therefore, the question whether the microbial phosphodiesterase, being not able to hydrolyze

8) R.O. Hurst, J.A. Little and G.C. Butler, *J. Biol. Chem.*, **188**, 705 (1951).

9) E. Volkin, J.X. Khym and W.E. Cohn, *J. Am. Chem. Soc.*, **73**, 1533 (1951).

10) J.F. Koerner and R.L. Sinsheimer, *J. Biol. Chem.*, **228**, 1049 (1957).

DNA, degrade polydeoxyribonucleotides with terminal 3'-monoesterified phosphate as well as the splenic phosphodiesterase still remains to be solved. RNA-depolymerases in the culture media of *Acrocylindrium* spec., *Cunninghamella echinulata*, *Pestalotia diospyri*, and *Pestalotiopsis funerea* were active at the acid pH and hydrolyzed DNA into 3'-mononucleotides, while DNA-depolymerases in them were active at the alkaline pH and hydrolyzed DNA into 5'-deoxymononucleotides. Therefore, these microorganisms seem to produce at least two enzyme systems, one of which catalyzes the decomposition of RNA and another degrades DNA.

Cunningham et al.¹¹⁾ described an enzyme in the culture medium of *Micrococcus pyogenes* that catalyze the degradation of DNA, yielding the 3'-deoxymononucleotides. Guschlbauer and Halleck¹²⁾ reported that *Pseudomonas aeruginosa* produce both extracellular and intracellular DNases. However, we failed to detect DNA-hydrolyzing enzyme in the culture media of both genera of micro-

organisms. It is likely that the discrepancy between these results is due to the difference in strains and culture conditions employed.

In the course of this study, we employed UPCA solution as a precipitant instead of 3 M TCA used by McDonald. The former precipitates the degradation product of DNA by DNase I⁴⁾ or DNase II¹³⁾ but the latter not. Therefore our screening tests detect no enzymes resembling DNase I or II and it is conceivable that these types of enzymes may be found in other microorganisms than those described above.

The details of the characters of microbial enzymes which concerned with the degradation of DNA are now being investigated.

Acknowledgements. The authors are indebted to Dr. S. Kuwada, Dr. K. Sato, Dr. S. Tatsuoka, Dr. M. Abe and Dr. T. Omura for their interests and encouragements. They wish to express their thanks to Dr. T. Hasegawa for supply of strains and to Dr. M. Yoneda and Mr. I. Suhara for the gift of 5'-nucleotidase and non-specific phosphomonoesterase and to Mr. S. Mori for his assistance.

11) L. Cunningham, B.W. Catlin and M. Privat De Garilhe, *J. Am. Chem. Soc.*, **78**, 4642 (1956).

12) W. Guschlbauer and F.E. Halleck, *Z. physiol. Chem.*, **323**, 121 (1961).

13) J.F. Koerner and R.L. Sinsheimer, *J. Biol. Chem.*, **228**, 1039, 1049 (1957).