Exonucleolytic Degradation of High-Molecular-Weight DNA and RNA to Nucleoside 3'-Phosphates by a Nuclease from B. subtilis

by

I. M. Kerr*, E. A. Pratt and I. R. Lehman

Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California

Received May 28, 1965

At present two nucleases, spleen phosphodiesterase (Heppel, Markham and Hilmoe, 1953) and the phosphodiesterase from Lactobacillus acidophillus (Fiers and Khorana, 1963), are known to degrade oligonucleotides completely to nucleoside 3'-phosphates. Neither of these enzymes, however, is active on high-molecular-weight polydeoxyribonucleotides (Schildkraut and Kornberg, personal communication). The present report is concerned with the isolation and characterization of a Ca^{++}-dependent extracellular nuclease from B. subtilis which degrades high-molecular-weight native and denatured DNA and RNA completely to nucleoside 3'-phosphates, the rate of degradation being greatest on denatured DNA. Degradation is almost exclusively exonucleolytic and at least in the case of denatured DNA appears to occur from the 5'-terminus of the polynucleotide chain. This enzyme should, therefore, prove a useful reagent in studies

---

* This investigation was conducted during the tenure of a Damon Runyon Cancer Research Fellowship.

on the composition, structure and biological activity of DNA and RNA.

The presence of an extracellular nuclease capable of degrading RNA to nucleoside 3'-monophosphates and possessing DNase activity has been reported by Nakai, Minami, Yamazaki and Tsugita (1965) for the Marburg strain of B. subtilis.

RESULTS

The nuclease was purified from the culture fluid obtained after growth of B. subtilis strain SB 19 for 24 hours at 30° in a yeast-extract veal-infusion broth medium. The starting material had an activity of 60 units*/ml on denatured DNA and 14 mg/ml of protein. After ammonium sulfate and acetone fractionation, chromatography on DEAE-cellulose yielded two peaks with activity on both native and denatured DNA (Figure 1). Each peak of activity retained its distinctive elution pattern on rechromatography on a second DEAE-cellulose column. Moreover, prior exposure of the load material to phase separation (Albertsson, 1962) or streptomycin sulfate fractionation had no effect on its chromatographic behavior. Further purification was aimed largely at the removal of the contaminating phosphatase activity from the DEAE-cellulose fraction which was eluted at higher salt concentration (Figure 1). Chromatography on Sephadex G200 reduced the phosphatase activity to a sufficiently low level as not to interfere with the use of this fraction as a reagent for the degradation of DNA to mononucleotides. Immediate concentration of this fraction on a DEAE-cellulose column yielded material (the Sephadex fraction) which was stable to storage for several months at 0° or -20°. It had a phos-

* As defined in the legend to Figure 1.

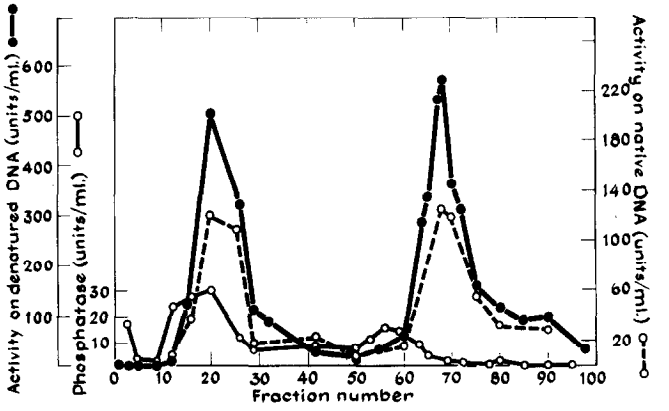

Figure 1. DEAE-cellulose chromatography of the B. subtilis nuclease.

Elution of fractions 1 to 8 was with 0.02 M and of fractions 9 to 43 was with 0.15 M Tris maleate buffer, pH 7.05 containing 10^{-3} M $MnCl_2$. Further elution was with a linear gradient 0.15 - 0.4 M Tris maleate buffer, pH 7.05 containing 10^{-3}M $MnCl_2$; the peak activity on denatured DNA eluted at a concentration of approximately 0.225 M.

One unit of DNase activity is defined as the amount catalyzing the production of 10 mμmoles of acid-soluble nucleotide in 30 minutes at 37°. For assays of DNase activity with denatured DNA as substrate the reaction mixture (0.3 ml) contained 20 μmoles of glycine buffer, pH 9.55, 0.5 μmole of $CaCl_2$, 20 mμmoles of P^{32}-labeled denatured E. coli DNA (Lehman, 1960) and 0.002 to 0.1 unit of enzyme. With native DNA as substrate the reaction mixtures were the same except for the replacement of the glycine buffer with Tris buffer, pH 8.0. The reaction mixtures were incubated for 30 minutes at 37° and assayed for production of acid-soluble P^{32} as described previously (Lehman, 1960). Concentrations of DNA are expressed as equivalents of nucleotide phosphorus.

Phosphatase activity was assayed by the release of Norit-non-absorbable P^{32} from P^{32}-labeled deoxyribonucleoside 3'-phosphates produced by digestion of denatured P^{32}-labeled E. coli DNA with the B. subtilis nuclease described here. Each assay (0.3 ml) contained 20 μmoles of Tris buffer, pH 8.0, 0.5 μmole of $CaCl_2$, 10 mμmoles of deoxyribonucleoside 3'-monophosphates and 0.05 to 0.5 unit of enzyme. One unit of phosphatase is defined as the amount which will produce 10 mμmoles of inorganic phosphate in 30 minutes at 37°.

---

phatase activity of 2 units/mg protein and specific activities of 1600 units/mg protein and 200 units/mg protein with denatured DNA and native DNA, respectively, as substrate. Unless otherwise stated this fraction was used in all the studies to be reported.

Characterization of the activity on DNA

Characterization of the products of digestion of DNA. Degradation of DNA by the B. subtilis nuclease was entirely (>99%) to nucleoside 3'-monophosphates. The results of an experiment in which the products of digestion of native and denatured DNA were each examined for susceptibility to E. coli alkaline phosphatase and to snake venom 5'-nucleotidase are presented in Table 1. After purification by paper chromatography and electrophoresis, the dTMP from the digest produced by 10 units of enzyme (Table 1) co-chromatographed with chemically synthesized

Table 1

Characterization of the products of digestion of native and denatured DNA by the B. subtilis nuclease

Experiment	Units* of enzyme	P^{32} DNA made acid-soluble	Nature of the acid-soluble P^{32}		
			Inorganic P^{32}	P^{32} susceptible to:	
				alkaline phosphatase	5'-nucleotidase
		%	%	%	%
1	1	100	1.9	96.5	–
2	2	100	2.4	97.0	–
3	10	100	6.0	93.5	1
4	1.25	100	6.9	92.0	0

* As assayed with the appropriate substrate.

In experiments 1, 2 and 3 the substrate was 10 mμmoles of denatured P^{32}-labeled E. coli DNA. In experiment 4 native DNA was used. Incubations were for 120 minutes at 37°. Assays for the release of phosphate by alkaline phosphatase and by 5'-nucleotidase were as described by Lehman, Roussos and Pratt (1962) and Linn and Lehman (1965b), respectively.

3'-dTMP in the n-propanol-ammonia-water system (Hanes and Isherwood, 1949) under conditions which gave a clear separation of 3'- and 5'-dTMP. The other three deoxyribonucleotides from the digest have not yet been characterized in this way.

Mode and site of attack. The complete degradation of DNA to mononucleotides observed with this enzyme suggested an exonucleolytic mode of attack. More definitive evidence was provided by an experiment in which the products at early stages in digestion of denatured DNA were assayed for susceptibility to alkaline phosphatase as an index of mononucleotide content (Table 2). Clearly, at between 4% and 15% digestion, these products were >95% mononucleotides. Very similar results were obtained in an analogous experiment using native DNA as a substrate.

The exonucleolytic attack appears to begin at the 5'-terminus of

Table 2

Susceptibility of products at early stages in digestion to alkaline phosphatase

Experiment	P^{32}-DNA rendered acid-soluble	Acid-soluble P^{32} susceptible to alkaline phosphatase
	%	%
1	4.2	97.4
2	7.0	97.5
3	15.5	97.2

20 mμmoles of heat-denatured P^{32}-labeled E. coli DNA were used in each experiment. Incubations were for 30 minutes at 37° with 0.1, 0.2 and 0.4 unit of enzyme.

the polydeoxyribonucleotide chain. The relative rates of release of total acid-soluble nucleotide and of P^{32} susceptible to alkaline phosphatase from denatured DNA labeled with a P^{32}-nucleotide at the 3'-terminus were compared for the B. subtilis nuclease and E. coli exonuclease I (Figure 2). The latter enzyme is known to degrade denatured DNA exonucleolytically liberating deoxyribonucleoside 5'-phosphates from the 3'-hydroxyl end (Lehman and Nussbaum, 1964). As shown in Figure 2, at a time when, for example, 10% of the DNA had been made acid-soluble by the action of exonuclease I, about 50% of the P^{32}-labeled 3'-terminal residues had been

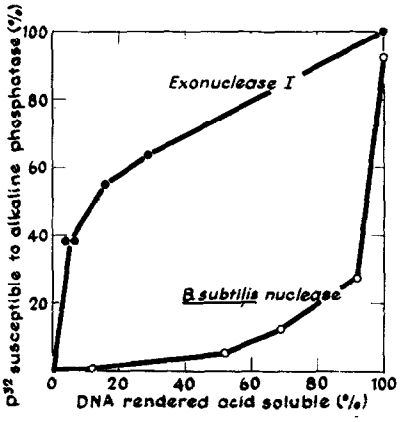

Figure 2. Release of nucleotides from the 3'-hydroxyl end of denatured DNA by E. coli exonuclease I and the B. subtilis nuclease. The 3'-hydroxyl terminal residues of E. coli DNA were labeled by incubation of the DNA with E. coli DNA polymerase in the presence of a single α-P^{32}-labeled deoxyribonucleoside triphosphate (dCTP) (Adler, Lehman, Bessman, Simms and Kornberg, 1958). The DNA was alkali-denatured prior to digestion. Digestion with E. coli exonuclease I was with the DEAE-cellulose fraction under the conditions described by Lehman and Nussbaum (1964) and with the Sephadex fraction of the B. subtilis nuclease under the conditions for denatured DNA (Legend to Fig. 1). Assays for the release of phosphate by alkaline phosphatase were as described by Lehman, Roussos and Pratt (1962) except that after incubation with the phosphatase residual DNA from the original digest was removed by precipitation with 0.175 N perchloric acid prior to Norit treatment.

The proportion of DNA rendered acid-soluble (abscissa) was based on the absorption at 260 mμ of the total 0.175 N perchloric acid-soluble fraction at different time points in digestion compared with that of a 100% digest of the DNA.

released as mononucleotides; at a comparable stage of digestion of the DNA by the B. subtilis nuclease, essentially no P^{32}-labeled mononucleotides were liberated. These results, therefore, are in accord with an exonucleolytic attack by the B. subtilis nuclease starting at the 5'-terminus of the polydeoxyribonucleotide chain.

As an alternative method of determining the initial site of attack, the products of digestion of the oligonucleotide d-TpTpT were examined. The initial products expected on sequential liberation of deoxyribonucleoside 3'-phosphates from the 5'-terminus of this oligonucleotide would be d-Tp and d-TpT, whereas degradation from the 3'-terminus would give rise initially to d-T and d-TpTp. On paper chromatographic analysis of the actual products obtained at early time points in such a digest only d-Tp and d-TpT were detected. The mononucleotide was characterized as d-Tp rather than d-pT on the basis of its insusceptibility to 5'-nucleotidase.

These results indicate a purely exonucleolytic mechanism for the enzyme; however, a very sensitive analytical ultracentrifuge assay using single-stranded M13 phage DNA "circles" (Mitra, 1965) demonstrated the presence of endonuclease activity in the Sephadex fraction. In view of this finding the endonuclease content of a more highly purified preparation of the nuclease obtained from the Sephadex fraction by electrophoresis on polyacrylamide gels (Ornstein, 1964; Davis, 1964) was investigated. On incubation of M13 phage DNA "circles" with an amount of this fraction sufficient to have produced 2.5% degradation to mononucleotides had the substrate been "linear" denatured E. coli DNA, no endonucleolytic scissions of the DNA "circles" were observed. After incubation with a 10-fold higher concentration of enzyme, however, no intact "circles" remained.

Characterization of the activity on RNA

RNase activity was assayed as described previously (Linn and Lehman, 1965a). The rate of degradation of E. coli ribosomal RNA was approximately half that observed with denatured DNA as substrate, was optimal at pH 9.5 and showed a requirement for Ca^{++}. On electrophoresis on polyacrylamide gels the activity on RNA migrated with that on DNA. These properties clearly distinguish this enzyme from the extracellular RNase crystallized from B. subtilis by Nishimura (1960). Preliminary results suggest that RNA also is attacked exonucleolytically with the liberation of nucleoside 3'-phosphates. Extensive digestion of E. coli ribosomal RNA rendered more than 95% of the RNA phosphate susceptible to alkaline phosphatase. On paper chromatography of such a digest the four ribonucleoside 3'-phosphates were the only products which could be detected. Furthermore, at 4%, 10%, 19% and 100% digestion of poly A, 3'-AMP was the only product detected on paper chromatography of the digests.

SUMMARY

A Ca^{++}-dependent extracellular nuclease which degrades high-molecular-weight native DNA, denatured DNA and RNA completely to nucleoside 3'-phosphates has been partially purified from cultures of B. subtilis. The rate of degradation is greatest on denatured DNA. Degradation is almost exclusively exonucleolytic and at least in the case of denatured DNA appears to occur from the 5'-terminus of the polydeoxyribonucleotide chain.

It seems likely that a single enzyme is responsible for the exonuclease activity on native and denatured DNA and on RNA. It is not yet clear, however, whether the low levels of phosphatase and endonuclease

present even in the most purified fractions are intrinsic activities of the exonuclease or represent contaminants. Further purification should provide a definitive answer to these questions.

REFERENCES

Adler, J., Lehman, I. R., Bessman, M. J., Simms, E. S. and Kornberg, A. (1958). Proc. Natl. Acad. Sci., Wash. 44, 641.

Albertsson, P. -Å. (1962). Arch. Biochem. Biophys. Suppl. 1, 264.

Davis, B. J. (1964). Ann. N. Y. Acad. Sci. 121, 404.

Fiers, W. and Khorana, H. G. (1963). J. Biol. Chem. 238, 2780 and 2789.

Hanes, C. S. and Isherwood, F. A. (1949). Nature (London), 164, 1107.

Heppel, L. A., Markham, R. and Hilmoe, R. J. (1953). Nature (London), 171, 1152.

Lehman, I. R. (1960). J. Biol. Chem. 235, 1479.

Lehman, I. R. and Nussbaum, A. L. (1964). J. Biol. Chem. 239, 2628.

Lehman, I. R., Roussos, G. G. and Pratt, E. A. (1962). J. Biol. Chem. 237, 819.

Linn, S. and Lehman, I. R. (1965a). J. Biol. Chem. 240, 1287.

Linn, S. and Lehman, I. R. (1965b). Ibid. 1294.

Mitra, S. (1965). Federation Proc. 24, 226.

Nakai, M., Minami, Z., Yamazaki, T. and Tsugita, A. (1965). J. Biochem. (Tokyo), 57, 96.

Nishimura, S. (1960). Biochim. et Biophys. Acta, 45, 15.

Ornstein, L. (1964). Ann. N. Y. Acad. Sci. 121, 321.