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An Extracellular Nuclease from *Serratia marcescens*

I. PURIFICATION AND SOME PROPERTIES OF THE ENZYME*

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SUMMARY

An extracellular nuclease was isolated from *Serratia marcescens* culture fluid which rapidly hydrolyzed DNA and RNA at approximately the same rate. The activity of the enzyme was purified 2600-fold with a final recovery of 25%. The ratio of enzymatic activity with RNA as substrate to that with DNA as substrate remained approximately constant throughout the purification and in all studies of the properties of the enzyme, leading to the conclusion that both activities are associated with a single protein. The enzyme required Mg^{++} or Mn^{++} for activity, and had a pH optimum of 8.5. Heating the enzyme to 44° or higher reduced enzymatic activity; after 40 min at 44°, all activity was abolished. The nuclease could be stored in glass-sealed ampules at 4° for up to 3 months without loss of activity. These properties, in conjunction with the specificity reported in the accompanying paper, make this enzyme potentially useful as a reagent for the study of nucleic acid structure and function.

Enzymes capable of catalyzing the depolymerization of nucleic acids have been isolated from a wide variety of sources. These nucleases are useful as reagents for the study of nucleic acid conformation, sequence, and biological function, and are also of interest as models for the study of protein structure and function.

As part of a search for new enzymes with properties and enzymatic specificities permitting their use as nucleic acid reagents, microorganisms capable of degrading nucleic acids were isolated from natural sources. In the course of these investigations, a powerful extracellular nuclease was isolated from an organism which later proved to have the same properties as a wild type strain of *Serratia marcescens*.

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This report deals with the purification and characterization of the activity of this nuclease, and an accompanying paper (1) describes its substrate specificity.

EXPERIMENTAL PROCEDURE

Materials

Torula RNA (B grade) obtained from Calbiochem was utilized for all RNase assays. DNase assays were performed with either calf thymus DNA (A grade) or salmon sperm DNA (A grade) obtained from Calbiochem. *p*-Nitrophenyl phosphate (A grade) and *p*-nitrophenyl thymidine 5′-phosphate (B grade) were products of Calbiochem. Bio-Gel P-6 (50 to 150 mesh) was purchased from Bio-Rad Laboratories, Richmond, California. DEAE-Sephadex was obtained from Pharmacia, and prepared according to the directions of the manufacturer. Crystalline bovine plasma albumin was obtained from Nutritional Biochemicals Corporation. Buffers and chemicals not otherwise specified were of reagent grade.

Amberlite IRC-50 (XE-64) was a product of Rohm and Haas; The Resinous Products Division, Philadelphia, Pennsylvania. The resin was prepared for use by a method similar to that of Hirs (2). It was washed thoroughly with distilled water by decantation, then equilibrated 3 hours in 0.5 M NaOH, washed with distilled water until nearly neutral, then equilibrated another 3 hours with 1 M HCl, and again washed with water. The resin was then packed in a column and washed with 1 M sodium phosphate, pH 6.8, until the effluent pH was nearly 6.8. Washing was continued with 0.01 M sodium phosphate, pH 6.8, until the effluent pH was the same. The column was stored in the same buffer containing 1 M NaCl.

Methods

Assay of S. marcescens Nuclease—The assay measures the conversion of RNA and DNA to fragments which are soluble in 2% perchloric acid, as followed by the appearance of acid-soluble material with an absorbance at 260 mμ. Each reaction mixture (0.5 ml) contained 0.5 mg of RNA or DNA, 50 mM Tris-HCl pH 8.2, 1 mM $MgCl_2$ (standard assay buffer), and 0.5 to 10 units of the enzyme which was diluted in standard assay buffer. Each reaction was started by the addition of 0.1 ml of properly diluted enzyme and the assay tubes were incubated at 37°. After 20 min, the reactions were stopped by the rapid addition of cold 4% perchloric acid (0.5 ml). The solutions were held on ice for 1υ

TABLE I

Purification of S. marcescens nuclease

Fraction	Volume	Total activity[a]	Total protein	Specific activity	Yield	Ratio of activity with RNA to DNA
	ml	*units*	*mg*	*units/mg*	%	
1. Culture fluid	1,000	800,000	4,400	182	100	1.3
2. Ammonium sulfate (40–80%)	24	480,000	300	1,590	60	1.2
3. Bio-Gel P-6	123	492,000	208	2,360	61	1.2
4. Amberlite IRC-50	305	381,000	1.22	312,000	48	1.3
5. Flash evaporation	12	225,000	0.72	312,000	29	1.2
6. Bio-Gel P-6	40	228,000	0.72	316,000	29	1.1
7. DEAE-Sephadex	53	201,000	0.42	475,000	25	1.2

[a] Activity assayed with *Torula* RNA as substrate.

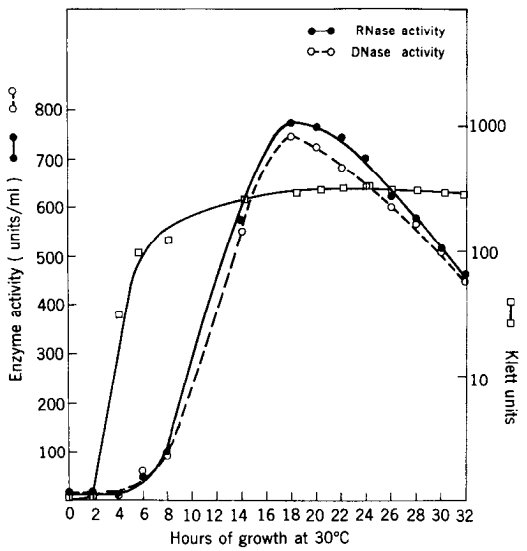

FIG. 1. Growth of *S. marcescens* and production of nuclease activity. Cells were grown under the conditions described in the text. Samples were removed at the times indicated and cell growth estimated with a Klett-Summerson photoelectric colorimeter equipped with a red filter. Enzyme activities were assayed as described under "Experimental Procedure."

min, then centrifuged 5 min at 8000 × *g*, and the supernatant fluids were removed and diluted 5-fold with water. The absorbance of these solutions at 260 mμ was read against a blank reaction in which buffer had been substituted for the enzyme. The change in absorbance was proportional to the amount of enzyme added when 5 to 75% of the substrate was solubilized (Fig. 3). This assay served as the basis for enzyme purification, and was used in all experiments describing the properties of the enzyme unless otherwise indicated.

A unit of RNase or DNase activity is defined as that amount of enzyme causing an increase of 1.0 in absorbance at 260 mμ in 20 min at 37°.

Specific activity is defined as enzyme units per mg of protein.

Other Assays—Phosphomonoesterase activity was determined with *p*-nitrophenyl phosphate substrate by a method similar to that of Garen and Levinthal (3). The substrate (0.2 ml of a 1 mM solution in standard assay buffer) was mixed with 0.1 ml of enzyme and incubated for 15 min at 37°. The reaction was stopped by the addition of 1 ml of 0.1 M NaOH, and the absorbance was measured at 410 mμ.

Phosphodiesterase activity was estimated in the same manner as phosphomonoesterase activity except that the substrate was *p*-nitrophenyl thymidine 5′-phosphate.

Protein was estimated by the method of Lowry *et al.* (4) using crystalline bovine plasma albumin as a standard.

Centrifugations were performed with a Sorvall RC2-B refrigerated centrifuge using an SS-34 rotor at 8,000 × *g* for assays, and the GSA rotor at 16,000 × *g* for preparative centrifugations.

Fractions were collected with an LKB Ultrorac type 7000 automatic collector.

All optical measurements were made with a Zeiss PMQ II spectrophotometer.

RESULTS

Purification of Enzyme

All procedures were carried out at room temperature and all centrifugations were performed at 16,000 × *g* for 15 min unless otherwise indicated. A summary of the purification procedure is given in Table I.

Growth of S. marcescens

A wild type strain ATCC 274 obtained from Dr. Clyde Willson was used in all experiments. Approximately 10^9 cells were inoculated into a 6-liter flask containing a culture medium of 24 g of Bacto-peptone (Difco) and 7.5 g of NaCl dissolved in 1500 ml of 50 mM Tris-HCl, pH 8.2, and 1 mM $MgCl_2$. Incubation was at 30° on a gyrotary shaker (New Brunswick Scientific) set at 100 rpm. Under these conditions, maximum nuclease activity was found after 18 to 22 hours of incubation (Fig. 1). Cells were harvested between 18 and 22 hours by centrifugation, and the deep red culture fluid (Fraction 1) was immediately subjected to ammonium sulfate fractionation.

Ammonium Sulfate Fractionation

One liter of the supernatant fluid was brought to 40% saturation by the rapid addition of 243 g of finely powdered ammonium sulfate. The solution was stirred until the salt dissolved and then was centrifuged. Preliminary experiments showed that good recovery of activity depended upon rapid completion of this step, and that the recovery was not improved by keeping the solution on ice. The red precipitate, which contained less than 5% of the total activity, was discarded. The pink supernatant fluid was brought to 80% saturation by the further addition of 343 g of ammonium sulfate, and the centrifugation was repeated. The 80% supernatant fluid, containing less than 1% of the total activity, was discarded, and the brown precipitate was carefully

drained and dissolved in 24 ml of standard assay buffer. The resulting deep brown solution (Fraction 2) could be stored several months at 4° with no apparent loss in activity.

Bio-Gel P-6 Fractionation

The ammonium sulfate fraction (24 ml) was desalted and brought to pH 6.8 by passage over a column (2.5 × 45 cm) containing 300 ml of packed Bio-Gel P-6 which had been equilibrated with 0.01 M sodium phosphate, pH 6.8. The column was eluted with the buffer at a flow rate of 2 ml per min. Fractions (20 ml) were collected and assayed for the presence of sulfate by precipitation with acidic $BaCl_2$ and for RNase and DNase activities and protein concentrations as described under "Methods." Sulfate-free fractions containing enzyme activity were combined (Fraction 3), and immediately subjected to ion exchange chromatography. This procedure gave total recovery of enzyme activity and a slight increase in specific activity because of loss of protein, and it was thus preferable to dialysis, which at this stage of purification resulted in a 30% loss in activity.

Chromatography on Amberlite IRC-50 (XE-64)

A column (5 × 12 cm) of 100 ml of prepared resin was equilibrated with 0.01 M sodium phosphate, pH 6.8 (column buffer), and washed with 2 liters of buffer to pack the resin. The enzyme recovered from the Bio-Gel fractionation was passed through the column which was then washed with 500 ml of column buffer at 2 ml per min. This procedure removed more than 99% of the total protein. The enzyme was eluted with 0.1 M NaCl in the column buffer. Fractions (20 ml) were collected and assayed for RNase and DNase activities and protein concentration as described under "Methods." Both enzyme activities were eluted in a single broad peak which corresponded to the major protein peak. All fractions containing enzymatic activity were pooled for immediate concentration since the colorless solution (Fraction 4) was unstable at 4°. The pooled fractions contained 78% of the activity applied to the column and represented a 1300-fold purification of the material.

Concentration by Flash Evaporation

Flash evaporation was performed with a Buchi Rotavapor at 37°. The pooled IRC-50 fractions (305 ml) were placed in a 1000-ml evaporating flask and concentrated to about 5 ml in less than 50 min. Good recovery of activity depended upon rapid completion of this step. The concentrated solution was removed from the flask and combined with several 2-ml distilled water washings to yield 12 ml of concentrated enzyme (Fraction 5). This concentrated fraction was stable and could be held at 4° for several months with no loss in activity.

Bio-Gel P-6 Fractionation

The concentrated enzyme solution was applied to a column (1.6 × 42 cm) containing 70 ml of packed Bio-Gel P-6 which had been equilibrated with 0.01 M sodium phosphate, pH 7.5. The enzyme was eluted in the same buffer at a flow rate of 2 ml per min. Fractions (10 ml) were collected and assayed for the presence of chloride ion by precipitation with silver nitrate and for nuclease activity and protein concentration as described under "Methods." The RNase and DNase activities were eluted together. Chloride-free active fractions were combined (Fraction 6) for immediate chromatography on DEAE-Sephadex, as the dilute enzyme was unstable at 4°.

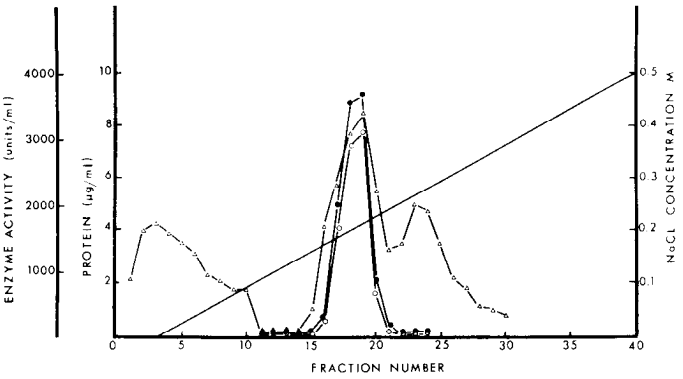

FIG. 2. Chromatography of *S. marcescens* nuclease on DEAE-Sephadex A-50. Enzyme and protein assays were performed as described under "Experimental Procedure." Protein, △——△; RNase activity, ●——●; DNase activity, ○——○.

Chromatography on DEAE-Sephadex A-50

A column (2.5 × 36 cm) was packed with 100 ml of DEAE-Sephadex A-50 equilibrated with 0.01 M sodium phosphate, pH 7.5. The entire Bio-Gel filtrate Fraction 6 (40 ml) was applied to the column and eluted with a linear gradient of NaCl (0.0 to 0.5 M in column buffer) in a total volume of 500 ml. Fractions (10 ml) were collected at a flow rate of 0.5 ml per min and assayed for activity and protein concentration as described under "Experimental Procedure."

An elution pattern obtained from the DEAE-Sephadex column is shown in Fig. 2. The RNase and DNase activities were eluted together as a single peak within one of the four broad protein peaks. The three fractions of highest specific activity were pooled (Fraction 7). These fractions contained 88% of the activity and 58% of the protein which had been applied to the column and represented a 1.5-fold purification of that material.

The Fraction 7 solution was distributed in 0.5-ml aliquots to glass ampules which were sealed and stored at 4°. This enzyme fraction was used for all further experiments involving the properties of the enzyme. The Fraction 7 enzyme represents a 2600-fold purification of the material in the cell culture fluid with a final yield of 25%.

It should be noted that throughout the purification procedure, the RNase and DNase activities were not separated, and the ratio of their activities was constant within the range of 1.1 to 1.3.

Properties of the Enzyme

The DEAE-Sephadex Fraction 7 enzyme was used in all studies to be described subsequently.

Rate and Extent of Hydrolysis of RNA and DNA Substrates

The time course of the hydrolysis of RNA and DNA by the *S. marcescens* nuclease is shown in Fig. 3. Conversion of substrate to acid-soluble products was linear for approximately 25 min, at which time 75% of the DNA and 85% of the RNA were acid-soluble. With both substrates, there was direct proportionality between the rate of hydrolysis and the enzyme concentration over the range of 4 to 40 mμg of protein under standard assay conditions as shown in Fig. 4.

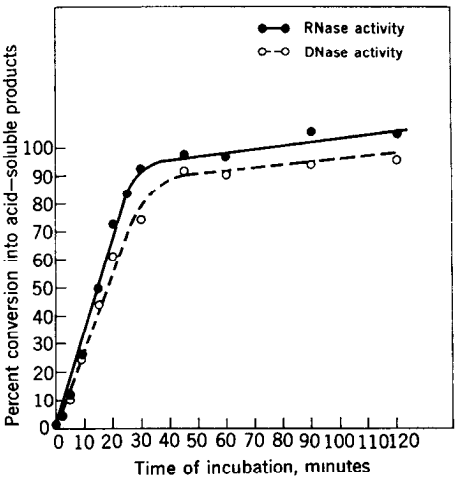

FIG. 3. Time course of hydrolysis of RNA and DNA by the purified *S. marcescens* nuclease. The standard reaction mixtures were scaled up to a volume of 6 ml. Each reaction was started by the addition of 1.5 ml of enzyme diluted 60-fold in standard assay buffer. At the indicated times, 0.5-ml aliquots were removed from each digestion mixture and immediately precipitated with 0.5 ml of cold 4% perchloric acid. The samples were then assayed as described under "Experimental Procedure." The acid-soluble absorbance following a 16-hour incubation was defined as 100% conversion, and all shorter time points are expressed as a percentage of this value.

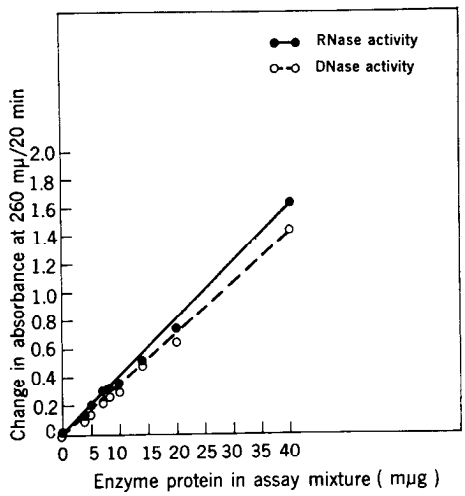

FIG. 4. Effect of protein concentration on the rate of hydrolysis of RNA and DNA. The enzyme was diluted 20- to 200-fold in standard assay buffer and assayed as described under "Experimental Procedure." Results are expressed as change in absorbance of acid-soluble material at 260 mμ in 20 min.

Absence of Contaminating Phosphomonoesterase and Phosphodiesterase Activities

As shown in Table II, the Fraction 7 enzyme had no detectable phosphomonoesterase or phosphodiesterase activities as measured by hydrolysis of *p*-nitrophenyl phosphate or *p*-nitrophenyl thymidine-5′-phosphate, respectively. Fractions 4 to 7 had less than 0.02% of the phosphomonoesterase activity and less than 0.003% of the phosphodiesterase activity which was present in the cell culture fluid. Furthermore, analyses of the products resulting from the *S. marcescens* nuclease hydrolysis of RNA and DNA, which are reported in an accompanying paper (1), show essentially no dephosphorylated dinucleotides (eluting before Peak 1) or trinucleotides (eluting at Peak 1), and little, if any, degradation of substrates smaller than tetranucleotides. This makes it unlikely that the enzyme is significantly contaminated with other nonspecific phosphomonoesterases or phosphodiesterases which do not hydrolyze the *p*-nitrophenyl substrates.

TABLE II

Purification of S. marcescens nuclease: absence of contaminating activities

Fraction	Total activity[a]		Recovery of activity	
	Phosphomonoesterase	Phosphodiesterase	Phosphomonoesterase	Phosphodiesterase
	units		%	
1. Culture fluid	49,000	371,000	100	100
2. Ammonium sulfate	4,300	17,800	8.8	4.8
3. Bio-Gel P-6	3,200	5,780	6.7	1.6
4. Amberlite IRC-50	<10	<10	<0.02	<0.003

[a] A unit of phosphomonoesterase or phosphodiesterase activity is defined as an increase in absorbance at 410 mμ of 0.1 per ml after 15 min at 37° in the assay systems described under "Experimental Procedure."

Stability

Fraction 7 enzyme stored at 4° was unstable and lost 50% of its activity in 1 week. However, enzyme samples sealed in 1-ml glass ampules could be stored up to 4 months of 4° with no detectable loss in activity. Rapid freezing of the sealed ampules in liquid nitrogen resulted in a 50% loss in activity, whereas slow freezing reduced the activity to 5% of the original level. Glass-sealed enzyme samples containing 50% glycerol, and those samples containing an 8-fold higher protein and salt concentration were stable to slow or rapid freezing, as well as to storage at 4°. Once the seal was broken, however, these samples gradually lost activity; only 50% of their original activities remained after 3 months. It should be noted that the stability of the enzyme under the various conditions employed was the same for both DNase and RNase activities. Enzyme samples sealed in glass ampules at 4° were used for all further studies.

pH Optimum

The rate of hydrolysis of RNA and DNA in buffers of varying pH value is given in Fig. 5. The enzyme was active from pH 7 to 10 with maximal activity around pH 8.5. Highest activities were found with Tris-HCl; activities in carbonate buffers were slightly lower. The maximal rate in phosphate buffer was only half that in Tris-HCl. The pH optimum and relative activity levels were the same for both RNA and DNA substrates, regardless of the type of buffer used.

Effect of Mg^{++} and Mn^{++}

The addition of Mg^{++} or Mn^{++} was required for enzyme activity. As shown in Fig. 6, maximal activity was obtained with 10 mM $MgCl_2$, whereas the maximal activity with $MnCl_2$ (1 mM) was only one-third that attained with $MgCl_2$. EDTA (1 mM) reduced both RNase and DNase activities by 30% in the presence of 1 mM $MgCl_2$; 0.1 M EDTA eliminated all enzyme activity.

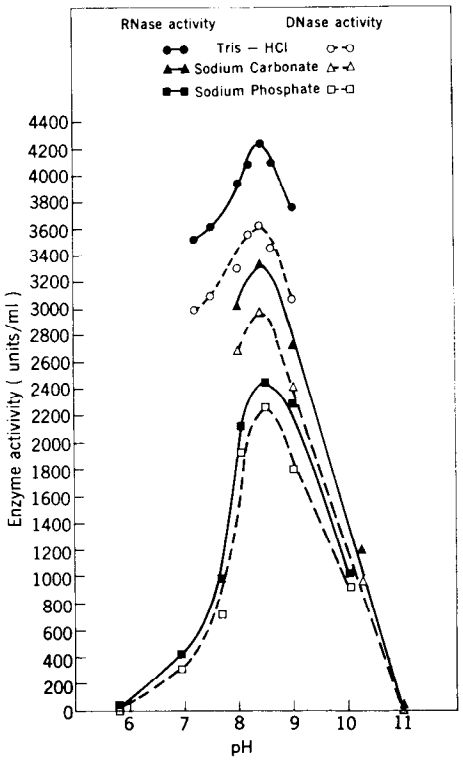

FIG. 5. Effect of pH on the activity of *S. marcescens* nuclease. Assays were performed as described under "Experimental Procedure." The Fraction 7 enzyme diluted 60-fold in distilled water was used for all determinations. The indicated buffers were added at a final concentration of 50 mM and all reactions were 1 mM in $MgCl_2$.

Effect of Inhibitors

Under standard assay conditions, iodoacetate (1 mM) had no effect on the enzymatic rate, whereas 1 mM mercaptoethanol and maleic acid reduced the activity by only 5 to 10%. Both RNase and DNase activities were progressively inhibited by $CaCl_2$ or NaCl concentrations greater than 10^{-4} M. In the presence of 1 mM $MgCl_2$, enzyme levels were reduced 75% by 0.1 M $CaCl_2$ or 1 M NaCl.

Effect of Temperature on Stability of Enzyme

The effect of 5 min of incubation at various temperatures upon the stability of the enzyme was measured. Enzyme samples were held for 5 min at different temperatures, chilled on ice, diluted, and then assayed as described under "Methods." Previous incubation of the enzyme at temperatures of 44° and above were all found to reduce enzyme activity. Both DNase and RNase activities were reduced to a similar extent by prior incubation at the elevated temperatures. Heating the enzyme for 5 min at 100° eliminated over 95% of its activity.

Rate of Heat Inactivation

The effect on both enzyme activities of incubation at 44° is shown in Fig. 7. Both activities were unstable at this temperature and declined steadily with the same rate constants of inactivation. Less than 5% of the initial activity remained after 40 min of incubation at 44°.

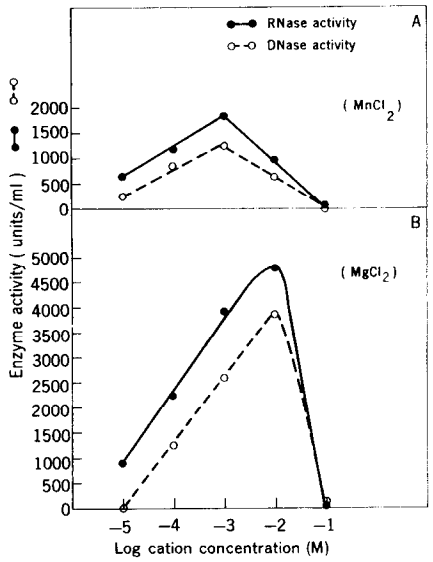

FIG. 6. Activation of enzyme activity by Mg^{++} and Mn^{++}. Fraction 7 enzyme diluted 60-fold in distilled water was used in all determinations. Assays were performed in Tris-HCl, pH 8.2, as described under "Experimental Procedure." Assay mixtures were brought to the indicated molarities by the addition of $MnCl_2$ (*A*) and $MgCl_2$ (*B*).

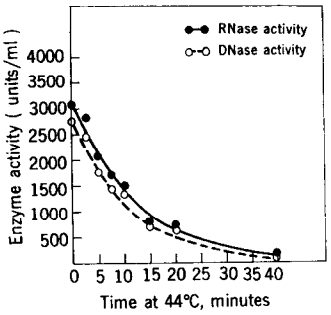

FIG. 7. Heat inactivation of the *S. marcescens* nuclease. A 10-fold dilution of the enzyme in standard assay buffer was held at 44°. At the indicated times aliquots were removed, thoroughly chilled, and diluted and assayed as described under "Experimental Procedure."

Evidence that Single Enzyme Catalyzes Hydrolysis of Both DNA and RNA

The experiments presented here are consistent with the idea that a single enzyme is responsible for the hydrolysis of both RNA and DNA substrates. This evidence may be summarized as follows.

Chromatographic Behavior—Both activities chromatograph together on all columns used, and the ratio of specific activities is essentially constant throughout the peak obtained after chromatography with DEAE-Sephadex. Throughout the purification procedure, the ratio of activity with RNA substrate to that with DNA remains nearly constant, varying from 1.1 to 1.3.

Kinetic Properties—Both substrates are hydrolyzed at nearly the same rate with the slight difference remaining constant throughout the time period studied (120 min).

pH Optima—Both activities exhibit maximal activity between pH 8.0 and 9.0 and parallel levels of activity at more acid and alkaline pH values with all buffers tested.

Effects of Cations, Sulfhydryl Reagents, and EDTA—Both

activities, which show parallel activation by Mn^{++} and Mg^{++} and inhibition by Ca^{++} and Na^{+}, are inactivated to the same extent by mercaptoethanol and maleic acid (5 to 10% inhibition), and are completely inactivated by 0.1 M EDTA under all conditions studied.

Heat Inactivation—Both activities are reduced at similar rates when the enzyme is heated at 44°.

Products Formed—As shown in the accompanying paper (1), the digestion products from both substrates are remarkably similar. In both cases, di-, tri-, and tetranucleotides terminating in 5′-phosphate are the major products formed, with a very small (less than 2%) proportion of mononucleotides also being produced.

Under no conditions studied were the two activities separable either physically or in terms of their properties. The evidence thus suggests that both activities are associated with the same protein.

DISCUSSION

The *S. marcescens* nuclease appears to be an extracellular enzyme. Under the growth conditions studied here, enzyme production increases during cell growth and reaches a peak just after the attainment of stationary phase, declining steadily thereafter. High nucleolytic activity is found in the cell-free culture fluid, and it seems unlikely that lysis of cells, which might be expected to occur during stationary phase, is responsible for the release of enzyme into the growth medium.

Under the best conditions of cell culture used in the purification procedure, enzyme protein accounts for only about 1 mg out of an apparent protein concentration of 3 g per liter of cell culture fluid. Most of this material is degraded peptone originally present in the growth medium, and is removed during the ion exchange chromatography with Amberlite IRC-50. An additional 2-fold purification is attained by the further chromatography of the enzyme on DEAE-Sephadex.

Although the maximum purification achieved is about 2,600-fold, it cannot be certain that a physically homogeneous preparation has been obtained, as the protein concentration of the DEAE-Sephadex fraction (8 μg per ml) was too low to permit application of the usual direct methods of physical characterization of proteins. It should be noted, however, that the specific activity of the best preparations of the *S. marcescens* nuclease is about 475,000 units per mg of protein. With the same assay procedures, under conditions optimal for each enzyme,[1] the specific activities of crystalline pancreatic ribonuclease, T_1 ribonuclease, and pancreatic deoxyribonuclease are about 275,000, 100,000, and 50,000 units per mg of protein, respectively.

Several other nucleases have been isolated from *S. marcescens*. Eaves and Jeffries (5) described a "nonspecific phosphodiesterase" similar to the one discussed here. Their enzyme was reported to be unaffected by repeated freezing and thawing or by sodium chloride concentrations as high as 0.2 M. These conditions are inhibitory to our enzyme in its purified form, although one cannot rule out differences in behavior due to variations in the purification procedure or the bacterial strain from which the enzymes were isolated. Furthermore, Eaves and Jeffries suggested that their enzyme most resembled micrococcal nuclease, an enzyme isolated from *Staphylococcus aureus* (6–8), in its properties. They did not, however, actually investigate the specificity of their enzyme. As reported in the accompanying paper (1), the enzyme described here differs from micrococcal nuclease in several important ways. (*a*) Micrococcal nuclease produces primarily mono- and dinucleotides after extensive digestion of RNA or DNA; (*b*) its products terminate in 3′-phosphate; (*c*) oligonucleotides containing adenylic or uridylic (thymidylic) acid residues are cleaved preferentially; and (*d*) it is more active on denatured than native DNA.

Several strains of *S. marcescens* have been investigated by Russian workers for possible nuclease activity (9) and a strain 41 (which is unavailable from the American Type Culture Collection) was chosen for further study (10). The Russian group separated two DNases on Sephadex G-100, one of which was reported to be a purine-specific deoxyendonuclease leaving products terminating in 5′-phosphate (11). An attempt was made to repeat their results using strain ATCC 274 and chromatographing the ammonium sulfate Fraction 2 on Sephadex G-100 according to their procedure. Only one peak of nuclease activity was found, with the RNase and DNase activities parallel, and in the usual ratio of 1.1 to 1.3. The single nuclease peak was free of phosphodiesterase and phosphatase activities. This inability to find a base-specific DNase activity may be due to differences in the bacterial strains from which the enzymes were isolated. In any case, the *S. marcescens* nuclease described here differs significantly from other enzymes reported from that organism, and thus represents another possible reagent for the study of nucleic acids.

[1] Unpublished experiments.

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