ISOLATION AND PROPERTIES OF AN EXOCELLULAR NUCLEASE OF *SERRATIA MARCESCENS*

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Abstract

Eaves, George N. (Wayne State University College of Medicine, Detroit, Mich.) and Charles D. Jeffries. Isolation and properties of an exocellular nuclease of *Serratia marcescens*. J. Bacteriol. **85**:273–278. 1963.—The exocellular nuclease of *Serratia marcescens*, isolated by anion-exchange chromatography on diethylaminoethyl-Sephadex, depolymerized deoxyribonucleic acid, ribonucleic acid, and the polynucleotide which is refractory to pancreatic ribonuclease activity. The enzyme was tentatively classified as a nonspecific phosphodiesterase. Magnesium was essential for activity, which was optimal at pH 8.8. The purified enzyme was completely inactivated by heating at 50 C for 15 min.

Exocellular nucleases produced by bacteria are usually reported as deoxyribonucleases and ribonucleases, depending upon the substrate used for their detection. For example, Masui et al. (1956) reported that *Staphylococcus aureus* and *Bacillus subtilis*, among others, secreted both types of nucleodepolymerases into the culture medium. Nishimura (1960) later crystallized an exocellular ribonuclease from *B. subtilis*, which implies that ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are degraded by distinct species of nucleolytic enzymes in this bacterium. In contrast, Cunningham (1959) reported that *S. aureus* secretes a phosphodiesterase which depolymerizes both DNA and RNA.

By use of the agar plate technique for determining the activity of microorganisms on nucleic acids, Jeffries, Holtman, and Guse (1957) demonstrated that *Serratia marcescens* could degrade both DNA and RNA. The present paper describes an investigation to determine whether *S. marcescens* secretes a single phosphodiesterase or both a ribonuclease and a deoxyribonuclease.

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Materials and Methods

Determination of enzymatic activity: ribonuclease assay. Commercial yeast RNA (Nutritional Biochemicals Corp., Cleveland, Ohio) was used as the substrate for the determination of ribonuclease activity, and was treated as follows. All manipulations were carried out at 2 to 4 C. The RNA was put into solution by the slow, dropwise addition of 3.5% sodium bicarbonate with constant stirring, and then precipitated by the addition of three volumes of cold ethanol, which had been adjusted to pH 4.5 with dilute acetic acid. After separation by centrifugation, the precipitate was dissolved in the original volume of water and dialyzed, with constant stirring, for 48 hr with repeated changes of distilled water. The RNA content of the solution was then determined by dry weights of triplicate 1-ml samples. The solution was diluted to give 10 mg per ml, dispensed in 1-ml amounts to Teflon-lined screw-cap vials, and stored in the freezer. The frozen solutions were thawed in a water bath at 30 C as needed.

The procedure used for the determination of ribonuclease activity, based on ultimate knowledge of the properties of the enzyme, was a slight modification of the ribonuclease assay developed by Anfinsen et al. (1954). To 1 ml of the substrate solution was added 0.5 ml of 0.4 M tris-(hydroxymethyl)-aminomethane-HCl (tris) buffer (pH 8.8) containing 0.04 M $MgCl_2$, and 0.5 ml of the enzyme solution. The reaction mixture was incubated in a water bath at 30 C for 30 min, after which the reaction was stopped by the addition of 0.5 ml of 0.75% uranyl acetate in

25% perchloric acid. The tubes were mixed by a single, rapid inversion and placed immediately in a refrigerated centrifuge (4 C) and spun for 10 min, during which a maximal centrifugal force of 6,600 × g was maintained for 6 min. Immediately after centrifugation, a 0.1-ml sample of the supernatant fluid was added to 3 ml of distilled water, and the absorbancy measured against distilled water at 260 mμ in a Beckman spectrophotometer, model DU. Correction was made for a reaction blank which contained all of the components of the reaction mixture, except that 0.5 ml of the buffer in which the enzyme was suspended was used in place of the enzyme solution.

Deoxyribonuclease assay. Deoxyribonuclease activity was detected by the same methods used for the determination of ribonuclease activity, except that the substrate solution contained 2 mg of highly polymerized, salmon sperm DNA (Mann Research Laboratories, Inc., New York, N.Y.) per ml of distilled water.

One unit of nuclease activity is defined as the activity of the enzyme which liberates the digestion product not precipitated by the uranyl acetate-perchloric acid reagent and gives an extinction change of 1 at 260 mμ under the conditions described.

Phosphodiesterase assay. The assay for phosphodiesterase activity was a modification of that used by Ostrowski and Walczak (1961). To 1 ml of 0.001 M bis(*p*-nitrophenyl)phosphoric acid (California Corporation for Biochemical Research, Los Angeles, Calif.) in distilled water was added 1 ml of 0.2 M tris buffer (pH 8.8) containing 0.02 M $MgCl_2$, and 0.1 ml of enzyme solution. The mixture was incubated at 30 C for 60 min, after which the reaction was stopped by the addition of 2.8 ml of 0.1 N NaOH to 0.21 ml of the reaction mixture. The resulting optical density was measured at 400 mμ (1 cm) with a Beckman spectrophotometer, model DU. Blanks were prepared by adding the enzyme solution to the substrate immediately before adding the NaOH.

To establish the efficiency of the phosphodiesterase-assay system, a positive control was set up as follows. A fresh mouse spleen was homogenized with water (100 mg per ml) in a Potter-Elvehjem homogenizer. The cellular debris was removed by centrifugation in the cold at 1,085 × g for 10 min. The supernatant fluid was used as the source of phosphodiesterase.

Proteinase assay. Proteinase activity was determined by a slight modification of the method used by Nomoto and Narahashi (1959). To 1 ml of 2% casein (Hammersten) in 0.03 M ammonium phosphate buffer (pH 7.5) was added 1 ml of enzyme solution, and the mixture was incubated for 10 min in a water bath at 30 C. The reaction was stopped by the addition of 2 ml of a protein-precipitating reagent containing 0.1 M trichloroacetic acid, 0.3 M acetic acid, and 0.2 M sodium acetate. This mixture was allowed to stand for 30 min at 30 C. The resulting precipitate was removed by filtration through Whatman filter paper no. 54. To 1 ml of this filtrate were added 5 ml of 0.4 M sodium carbonate and 1 ml of diluted (1:5) Folin-Ciocalteau's reagent. After 30 min at 30 C, the optical density of the resulting colored solution was measured at 670 mμ on a Coleman Junior Spectrophotometer, model 6A. The blank was made by adding the enzyme solution, or the diluting buffer, after the precipitating reagent was added to the casein solution.

One unit of proteinase activity is defined as the activity of the enzyme which liberates the digestion product not precipitated by the above reagent and gives an extinction change of 1 at 670 mμ under the conditions described.

Phosphomonoesterase assay. Acid and alkaline phosphatase activities were determined by the method of Lowry (1957).

Amylase assay. Amylase activity was determined by the method of Bernfeld (1955), using soluble starch as the substrate.

Determination of protein. During purification of the enzyme, protein concentrations were determined from measurements of absorbancy at 280 and 260 mμ with a Beckman spectrophotometer model DU, according to the method of Warburg and Christian (1942).

Basal medium. The basal broth, a modification of that used by McCarty (1952), contained the following constituents: 0.5% Casamino Acids (vitamin-free), 0.5% dextrose, 0.2% NaCl, 0.5% K_2HPO_4, 0.005% $FeSO_4$, and 0.05% $MgSO_4$. Basal agar medium was made by adding 1.5% agar to the basal broth.

Isolation of enzyme: (step 1) *preparation of crude extract.* Basal agar medium (pH 7.2) was dispensed in 750-ml amounts in Pyrex baking dishes and inoculated by lattice-form cross

streaking with a cotton swab saturated with *S. marcescens* strain 1783-57 (Communicable Disease Center, Atlanta, Ga.) which had been grown in basal broth for 24 hr at 30 C. After around 70 hr of incubation at 30 C, the cells were removed from the surface of the medium with the aid of glass spreaders, and the plates were frozen. The following day, the frozen agar was thawed at room temperature and the expressed fluid collected by filtration through a Büchner funnel.

(Step 2) *Concentration with ammonium sulfate.* The pH of the expressed fluid was adjusted to 5.5 with 1 N HCl, and the solution was adjusted to 0.8 saturation with solid ammonium sulfate (561 g per liter). The solution was left in the cold (4 C) overnight. The resulting precipitate was collected by centrifugation at 23,500 × *g* for 1 hr in the cold, resuspended in a small volume of water, lyophilized, and stored in the freezer.

(Step 3) *Fractionation on a diethylaminoethyl (DEAE)-Sephadex A-50 column.* The lyophilized material was dissolved in 0.02 M sodium phosphate buffer (pH 8.1) and dialyzed for 4 hr in the cold against the same buffer. DEAE-Sephadex A-50 medium (Pharmacia, Uppsala, Sweden) was activated as instructed by the manufacturer and equilibrated with 0.02 M sodium phosphate buffer (pH 8.1). Fractions were collected by a fraction collector equipped with a drop-counting unit (Research Specialties Co., Richmond, Calif.). The fractions were assayed for the presence of protein, proteinase, and nuclease with both DNA and RNA as substrates.

RESULTS

Table 1 summarizes the results of the isolation procedure in which the nuclease was purified 234-fold with a recovery of 22% of the enzyme activity. Figure 1 shows the isolation of the nuclease by chromatography on DEAE-Sephadex A-50. Although proteinase and nuclease activity were separated by the chromatographic procedures described, only the nuclease was actually purified significantly; the proteinase was purified only sevenfold. There was a single peak of nuclease activity when both DNA and RNA were used as substrates. This single peak of activity (fractions 6 and 7) was subsequently chromatographed on a cation exchanger SE-Sephadex C-50 medium (Pharmacia) at pH 5.5 (0.02 M acetate buffer). The enzyme was strongly adsorbed to this exchanger and could not be eluted by a continuous pH gradient (established by eluting with 0.02 M sodium acetate up to pH 7.7). The enzyme could be eluted by increasing the ionic strength with a NaCl gradient; however, the activity against DNA and RNA was not separated.

Although traces of exocellular amylase were detected around the growth of *S. marcescens* by the standard plate technique (Society of American Bacteriologists, 1957), the solution of exocellular proteins subjected to chromatography contained no detectable amylase activity. Similarly, there was no detectable acid nor alkaline phosphatase activity in the starting material.

The properties of the enzyme were determined with the chromatographically isolated enzyme (step 3). Figure 2 shows the effect of magnesium on nuclease activity with RNA as substrate. The nuclease activity was not diminished more than 10% within the range of 0.05 to 0.005 molar concentration of magnesium. However, the

TABLE 1. *Isolation of the exocellular nuclease of Serratia marcescens*

Step	Preparation	Total vol	Protein	Nuclease activity	SA*	Yield
		ml	*mg/ml*	*units/ml*		*%*
1	Fluid expressed from agar culture	2,530.0	0.92	1.68	1.8	100
2	$(NH_4)_2SO_4$ concn	120.0	3.22	33.60	10.4	94.8
	Lyophilized sample, dissolved in buffer	3.1	3.32	34.53	10.4	
3	Chromatography on DEAE-Sephadex A-50					
	Fraction 6	3.7	0.015	6.40	420.7	22.1 } 44.9
	Fraction 7	3.7	0.020	6.60	330.0	22.8 }

* Specific activity, expressed as units/mg of protein.

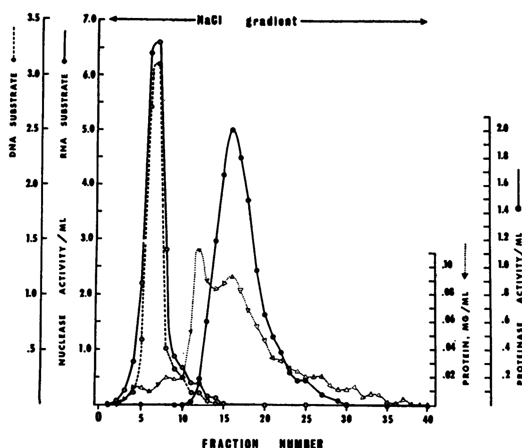

FIG. 1. *Anion-exchange chromatography on DEAE-Sephadex A-50 of extracellular proteins of Serratia marcescens. Solution of extracellular proteins, 10.27 mg in 3.1 ml of 0.02 M sodium phosphate buffer (pH 8.1), placed on a column (10 by 185 mm). Tube 1 contained the effluent collected during addition of protein solution. An ionic-strength gradient was established by placing 0.02 M sodium phosphate buffer (pH 8.1) in the mixing vessel (60 ml), and buffer containing 0.6 M NaCl in the reservoir. Flow rate was approximately 30 ml per hr. Fraction volume was 3.7 ml. Nuclease and proteinase activities expressed in units per ml.*

reaction was limited considerably by an insufficient or an excessive amount of magnesium. A similar result was observed with DNA as the substrate. The optimal concentration of magnesium for activity against RNA was 0.01 M.

To determine whether cations other than magnesium would fulfill the cofactor requirements, the following compounds were substituted in final concentrations of 0.005 and 0.01 M: $MnCl_2$, $FeCl_3$, $CuCl_2$, $ZnCl_2$, $CaCl_2$, NaCl, KCl, and $PbCl_2$. Manganese would replace magnesium; however, the addition of 0.01 M $MnCl_2$ to the reaction mixture resulted in less than half as much activity as was obtained with 0.01 M $MgCl_2$. This observation may actually reflect the affinity of tris buffer for manganese, rather than a relationship between manganese and the enzyme reaction. No other cation tested would substitute for magnesium. Similarly, there was no activity in reaction mixtures containing 0.0125 and 0.025 M ethylenediaminetetraacetic acid or distilled water in the absence of cations.

The optimal pH was determined by using the following buffers in a final concentration of 0.1 M: sodium acetate, sodium phosphate, tris, and sodium carbonate. To eliminate the possibility of buffer effect, the last pH unit of a given buffer was the same as the first pH unit of the subsequent buffer. The effect of pH on nuclease activity, with both DNA and RNA as substrates, is shown in Fig. 3. The optimal pH was around 8.8 with both substrates.

Stability of the purified enzyme to heat was determined at pH 5.0 (0.05 M sodium phosphate buffer) and pH 8.8 (0.05 M tris buffer). Samples containing 0.01 mg of protein per ml were exposed to temperatures ranging from 30 to 90 C for 10 min at pH 5.0 and 8.8. All of the activity was lost after heating at 60 C for 10 min at both pH values. Subsequently, the rate of inactivation in 0.02 M sodium phosphate buffer (pH 8.0) was determined at 50 C. Table 2 shows the rate of inactivation with DNA and RNA as substrates. The enzyme was completely inactivated by heating at 50 C for 15 min. The enzyme is, however, quite stable at refrigerator temperatures. A sample of nuclease in 0.02 M sodium phosphate buffer at pH 8.0 retained 70% of its original activity after 4 weeks of storage in a refrigerator. In addition, the enzyme was little affected by repeated freezing and thawing.

The possible effect of ionic strength on the activity of the nuclease was evaluated by adding NaCl to the assay reaction mixture in final concentrations of 0.01, 0.025, 0.02, 0.1, 0.15, and 0.2 M. Enzyme activity did not appear to be significantly affected by these variations in ionic strength.

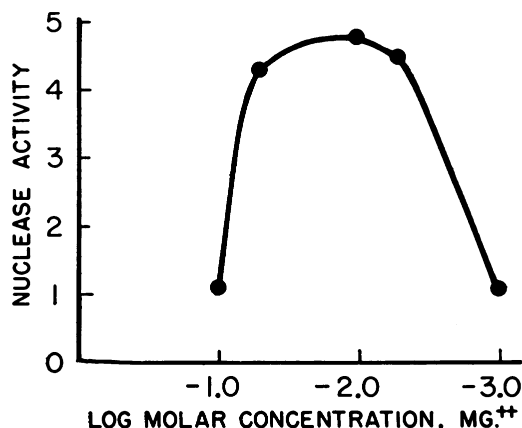

FIG. 2. *Effect of magnesium concentration on the activity of Serratia marcescens nuclease against ribonucleic acid.*

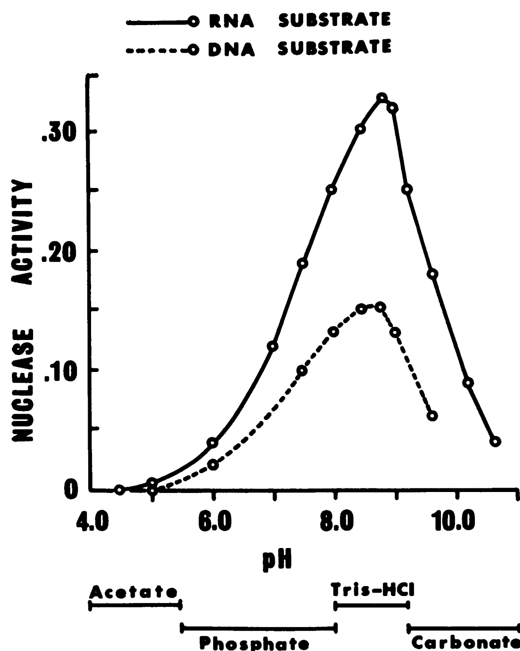

FIG. 3. *Effect of pH on nuclease activity.*

TABLE 2. *Rate of inactivation of purified Serratia marcescens nuclease at 50 C*

Time	Relative per cent activity	
	DNA substrate	RNA substrate
min		
0	100	100
5	11	18
10	4	3
15	0	0

To ascertain the range of activity, the purified nuclease was tested on both DNA and RNA which had been heated in boiling water for 10 min and cooled rapidly (in an ice bath) to afford permanent separation of the single strands comprising the double-stranded nucleic acid helix. In addition, "ribonucleic acid-core" (Worthington Biochemical Corp., Freehold, N.J.) and bis(*p*-nitrophenyl)phosphoric acid were used as substrates. "Ribonucleic acid-core" was depolymerized; however, there was no activity against bis(*p*-nitrophenyl)phosphoric acid. No increase in activity was obtained when heat-denatured DNA and RNA were used as substrates. The nuclease required magnesium for its ability to degrade DNA as well as RNA. Calcium would not substitute for magnesium, at pH 6, 7, or 9, in the degradation of either DNA or RNA.

DISCUSSION

The results of this study indicate that the enzymatic degradation of DNA and RNA by *S. marcescens* is by a single enzyme. The nuclease obtained in a highly purified state from column chromatography was active against DNA, RNA, and "RNA-core" (the polynucleotide which is refractory to pancreatic ribonuclease activity). In addition, the pH optimum, the requirement for magnesium, and the heat lability further indicated that the enzymatic activity was associated with a single entity.

Although many different bacterial nucleases have been reported, few have been adequately described. The general properties of the reported nucleases show that there is considerable variation among the ribonucleases and deoxyribonucleases, even those from the same species, e.g., the group A streptococci (Wannamaker, 1958). Whereas most bacterial nucleases have been reported as ribonucleases or deoxyribonucleases, only the nuclease of *S. aureus* (Cunningham, 1959) has been described as a nonspecific phosphodiesterase. The properties of the *S. marcescens* phosphodiesterase show that it is closest in its properties to the "micrococcal nuclease." For example, both nucleases attack DNA, RNA, and "RNA-core," both require a divalent cation for activity, and both have optimal activity at about the same pH. The nuclease of *S. marcescens* differs from the *S. aureus* nuclease only by its requirement for magnesium, rather than calcium, and its heat lability. Neither of the enzymes could degrade bis(*p*-nitrophenyl)phosphoric acid.

It should be noted that the nuclease of *S. marcescens* can degrade DNA and RNA in the absence of inorganic phosphate. This observation eliminates the possibility of a polynucleotide phosphorylase as observed in *Azotobacter vinelandii* by Grunberg-Manago, Ortiz, and Ochoa (1956) and in *Micrococcus lysodeikticus* by Beers (1957).

The properties of the *S. marcescens* nuclease suggest that the enzyme is a nonspecific phosphodiesterase. Such a classification, which is dependent upon the assumption that there is a single enzyme rather than two specific nucleases, can be justified by the following observations:

(i) anion-exchange chromatographic isolation resulted in a single peak of nuclease activity against both DNA and RNA; (ii) rechromatography on a cation exchanger resulted in a single peak of activity against both substrates; (iii) the purified enzyme depolymerized "RNA-core" in addition to DNA and RNA; (iv) the optimal pH for the hydrolysis of DNA and RNA was the same; (v) the hydrolysis of DNA and RNA by this enzyme required magnesium or manganese; and (vi) the rate at which the enzyme was inactivated by heat was the same for both nucleic acid substrates.

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