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Structural and Enzymatic Properties of the Extracellular Nuclease of *Micrococcus pyogenes*

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The extracellular nuclease produced during the growth of *Micrococcus pyogenes* has been isolated as an essentially homogeneous protein which is capable of hydrolyzing phosphate diester bonds in both yeast RNA and calf thymus DNA. The enzyme has a pH optimum of approximately 8.6 – 8.8 with RNA as substrate. Preliminary studies indicate a sedimentation coefficient of 1.7 S and an isoelectric point at pH 7.0 – 7.5. The results of amino acid analyses permit the calculation of a rough molecular weight of 11 000 – 12 000 based on 2 histidine residues per molecule. On this same basis, the protein contains approximately 100 amino acid residues per molecule, including one residue of tryptophan. The polypeptide chain is free of SH groups and of disulfide bonds suggesting that the calcium ions which are required for activity may be involved in the stabilization of the tertiary structure of the enzyme.

The deoxyribonuclease found in the culture medium of *Micrococcus pyogenes* was originally purified by Cunningham and his colleagues[1–3] and shown to yield mainly 3'-mononucleotides. Further studies by Reddi[4–6] and by Privat de Garilhe *et al.*[7] have confirmed the calcium requirement of the enzyme and have further demonstrated that the partially purified protein preparation exhibits activity towards both RNA and DNA. Alexander *et al.*[8] have purified the enzyme extensively and have presented detailed studies on specificity. These studies, in contrast to some of the data presented by Privat de Garilhe *et al.*[7], strongly suggest that a single enzyme is responsible for both ribonuclease and deoxyribonuclease activities.

The relatively small size of the protein makes it ideally suited for a study of

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covalent structure in relation to activity. Such investigations are of special interest because of the possibility that the catalytic center of the protein cannot distinguish between the ribose and deoxyribose moieties of the substrate molecules. Thus a comparison of the sequence and tertiary structure of the micrococcal nuclease with that of bovine pancreatic ribonuclease, whose structure is known, may lead to useful information on the three-dimensional configurations and mechanisms of action of the respective catalytic sites.

The present studies permit us to state, with some assurance, that the same protein does, indeed, attack both substrates. The enzyme consists of a single polypeptide chain, devoid of disulfide cross linkages. The nuclease has been purified to a point where it appears essentially homogeneous in the ultracentrifuge and during free electrophoresis, and calculations based on amino acid analysis yield a molecular weight of approximately 12 000.

EXPERIMENTAL

Growth of organisms. Micrococcus pyogenes (Staphylococcus aureus, Strain V-8)[9] was grown in 100–150 l lots, with vigorous bottom aeration, of culture medium "CCY"[9]. This medium contains (per l) 30 g oxoid casamino acids, 20 g sodium β-glycerophosphate, 10 g sodium lactate, 2.45 g Na_2HPO_4, 0.4 g KH_2PO_4, 6.4 mg $FeSO_4 \cdot 7H_2O$, 6.4 mg citric acid, 10 mg $MnSO_4 \cdot 4H_2O$, 20 mg $MgSO_4 \cdot 7H_2O$ and 20 % (v/v) of oxoid yeast diffusate (pH 7.2 – 7.4). Growth was carried out at 37°C overnight, with vigorous stirring. We wish to express our gratitude to the Microbiological Research Establishment, Porton, Wilts, England, for growing and harvesting the organisms. The culture medium was cooled to below 10°C, clarified by centrifugation, and the supernatant sterilized by filtration. $(NH_4)_2SO_4$ was stirred into the filtrate until the solution was saturated and the suspension was allowed to stand overnight at room temperature. The bulk of the precipitate rose to the surface and, after the solution below had been siphoned off, was collected by filtration after addition of suitable amounts of Hyflo Supercel. Phosphate buffer (0.05 M, pH 6.8 – 7.0) was added to the filter cake until the ammonium sulfate concentration was reduced to 2.2 M. The suspension was filtered and the residue was re-extracted with 2.2 M ammonium sulfate in the same phosphate buffer. The nuclease was precipitated from the combined extracts by saturation of the solution with ammonium sulfate and, after the addition of Hyflo Supercel, the precipitate collected by filtration. These preliminary steps yielded approximately two-thirds of the total units of nuclease activity in the original culture filtrate. The filter cake from approximately 2000 l of culture medium, containing the precipitated enzyme together with the Hyflo Supercel, was shipped to this laboratory for further purification.

Ribonuclease activity. Enzyme solutions were diluted with sodium borate buffer, 0.1 M, pH 8.8, in such a manner that 5 to 10 μl sufficed for each assay. The aliquot taken was added to a solution containing 0.5 ml 0.4 % yeast RNA, prepared according to Crestfield, Smith and Allen[10], 0.1 ml 0.1 M $CaCl_2$ (calcium ions are an absolute requirement), and 0.4 ml borate buffer, pH 8.8, 0.1 M. The RNA was also made up in the same buffer. Following incubation at 37°C for 30 min, 0.5 ml uranyl acetate (0.75 % in 25 % perchloric acid) were added. The tubes were centrifuged to remove precipitated oligonucleotides and undigested RNA, 0.1 ml of the supernatant were diluted to 3.1 ml with water, and the optical density at 260 mμ was measured. One unit of enzyme activity is defined as that amount which produces, under the above conditions, a change of absorbancy of 1.0 at 260 mμ.

Deoxyribonuclease activity was determined by a modification of the method described by Alexander *et al.*[8]. Five to 10 μl of suitable diluted enzyme solution were added to a solution containing 0.1 ml substrate solution (calf thymus DNA, preparation "Simmons B"[11], 3 mg/ml in a solution which is 0.005 M in NaCl and 0.05 M in sodium borate, pH 8.8). After incubation at 37°C for 30 min, 0.5 ml perchloric acid was added. The tubes were then centrifuged and the supernatant was diluted and read at 260 mμ as described by Alexander *et al.*[8]. Units of DNase activity are defined as for RNase activity above. DNase activity (and, to a smaller extent, RNase activity as well) is strongly influenced by the nature of the buffer with which the enzyme is diluted prior to sampling for assay. Thus citrate buffer may yield much higher

(*ca* 6-fold) activities than phosphate and borate buffers. This effect does not influence the *relative* activities within any single experiment but can cause marked variations in the results obtained from experiment to experiment since the previous history of the samples may have a strong influence on the apparent activities measured. The variations due to ionic environment are being investigated further.

Chromatography was performed at 5°C on CM-cellulose (California Biochemical Institute, Cellex-CM, *ca* 0.7 meqv/g) by a modification of a method described earlier[12] (see below). Relative protein concentrations were estimated by spectrophotometric measurements in the Zeiss spectrophotometer at 280 mμ. Titration of the native enzyme for SH group content was carried out as described by Boyer[13], over a wide range of enzyme concentrations (0.01—0.1 μmoles/2.5 ml).

Samples of protein which appeared to be homogeneous on the basis of physical measurements and estimations of specific enzyme activities of effluent fractions from chromatographic columns were subjected to reduction and alkylation[14]. After removal of urea and mercaptoethanol from the solution of reduced, alkylated protein by dialysis against water, the solutions were lyophilized. Weighed samples of the dried protein were hydrolyzed in constant boiling 6 N HCl in evacuated, sealed tubes at 110°C and the hydrolysates, after removal of HCl, were analyzed for amino acid content in the automatic amino acid analyzer (Beckman-Spinco Model 120B) according to the procedure of Spackman *et al.*[15] Samples were acid hydrolyzed for 20 and 44 h. The values obtained for those amino acids which are unstable, or slowly released, under conditions of acid hydrolysis[16] have not been corrected in the present paper.

Samples of the dried protein, after exposure to conditions of reduction and alkylation as described above, were digested with trypsin to yield peptides for "fingerprinting". In these digestions, 3—4 mg of protein were dissolved in 0.35 ml 0.2 M NH_4HCO_3, pH 8 and treated for 2 h at 37°C with 0.07 mg trypsin which had previously been exposed to limited amounts of diisopropylfluorophosphate to inactivate the bulk of contaminating chymotrypsin-like activity[17]. Peptide maps were prepared by chromatography in butanol-acetic acid-water of aliquots of the digests equivalent to approximately 1 mg of protein, followed by electrophoresis at pH 3.6 in the "High Voltage Electrophorator" (Model D, Gilson Medical Electronics, Middleton, Wisconsin)[18]. The resulting maps were stained either with 0.025 % ninhydrin in absolute ethanol, with Sakaguchi reagent, for the location of arginine residues, or with the Ehrlich stain for tryptophan residues.

Purification. CM-cellulose was successively washed with 1 N NaOH, 3 N HCl, and water. Water washing was continued until a negative test for chloride ions was obtained. The ion exchanger was then equilibrated with 0.05 M Na-PO_4 buffer, pH 6.1. Columns having the dimensions 2.5 $\times$ 30 cm were prepared and a solution of the crude ammonium sulfate precipitate, described above, was applied in a small volume after preliminary dialysis against the 0.05 M buffer and removal of Hyflo Supercel. The column was washed with this same buffer until the effluent exhibited an extinction at 280 mμ of less than 0.05. A closed two-compartment gradient was then begun in which potassium phosphate, 0.2 M, pH 8.0, was added to the mixing chamber which contained the 0.05 M buffer at pH 6.1. Fig. 1 shows the typical elution diagram of a preparative run. The nuclease activity emerged last from the column at an effluent pH value of approximately 7.1—7.3. The phosphate concentration at this point was approximately 0.15 M. The tubes containing the active component were pooled and the combined solution diluted with 8 parts of distilled water and 1 volume of 0.1 M $CaCl_2$, in that order. The copious precipitate of calcium phosphate was collected by centrifugation and the supernatant, devoid of all but traces of activity, was discarded. The precipitate was suspended as a slurry in 0.1 M citrate buffer, pH 7.5 and 0.1 M sodium ethylenediaminetetraacetate (EDTA) was added (0.8 ml/40 ml final volume). The suspension was transferred to a dialysis bag and dialyzed over night against two 1 l changes of citrate buffer-EDTA prepared as described above. The resulting slightly turbid solution was centrifuged in the Servall centrifuge for 1 h at 10 000 rpm. The supernatant solution was saturated with ammonium sulfate and allowed to stand for 20 h at 5°C. The precipitate which formed was collected by centrifugation, dissolved in a minimum volume of 0.05 M citrate buffer, pH 7.5 and dialyzed against the same buffer overnight. The purification procedure described above in general permits the recovery of about 50 % of the enzyme activity present in the original crude ammonium sulfate fraction. A more exact appraisal of the recoveries at each stage of purification will be presented in a later communication.

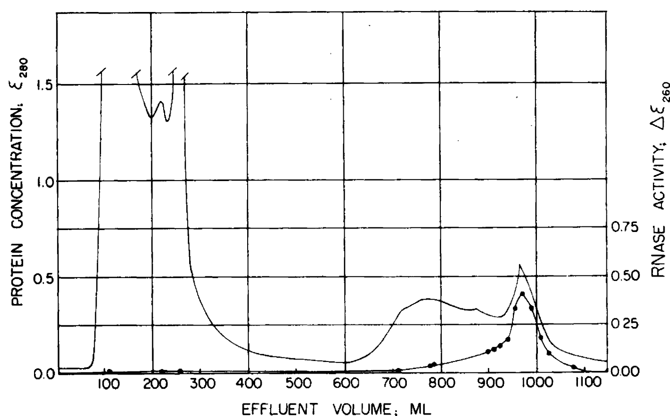

Fig. 1. Elution diagram from a preparative chromatographic purification, on CM-cellulose, of a crude, dialyzed ammonium sulfate precipitate. The precipitate, containing the nuclease activity, was prepared from the culture medium as described in the text, after removal of *Micrococcus pyogenes* cells. The bottom curve represents the levels of ribonuclease activity in the effluent fractions, and the upper curve the protein concentration as estimated by extinction measurements at 280 mμ.

RESULTS AND DISCUSSION

The enzyme, at this point of purification, appears to be essentially homogeneous and suitable for studies of structure and enzymatic specificity. Patterns obtained on such material in the Spinco Ultracentrifuge, Model E, are shown in Fig. 2. No traces of heavy components were seen during the early stages of centrifugation. The same preparation employed in the ultracentrifuge run shown in Fig. 2 was subjected to free boundary electrophoresis and the final pattern obtained after 20 h of migration is shown in Fig. 3. The schlieren diagram shows two small components in a total amount of perhaps 5 % of that of the major peak

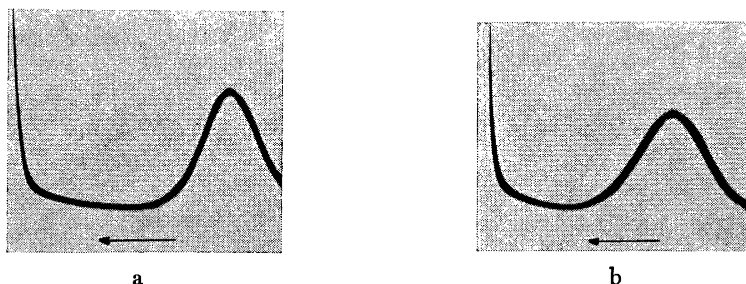

Fig. 2. The sedimentation behaviour of purified nuclease: a) 75 min after reaching final rotor speed, bar angle 55°, 21°C, 59 780 rpm; b) 114 min, bar angle 50°, 21°C. The protein was present at a concentration of 7 mg/ml, in a buffer which was 0.025 M sodium citrate, 0.05 M NaCl, pH 7.5. The arrows indicate the direction of sedimentation.

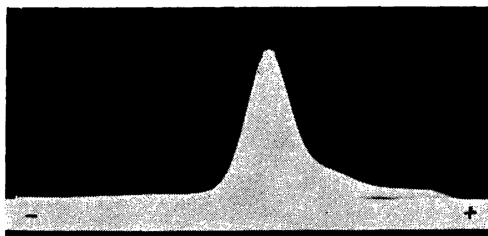

Fig. 3. Electrophoretic pattern obtained on the same preparation employed for the sedimentation experiment shown in Fig. 2. 0.05 M sodium citrate buffer, pH 7.5, 13 mA, 3.4 V/cm, 1200 min, protein concentration 6.5 mg/ml. A sample, withdrawn from the portion of the cell containing the two small components on the cathodic side, was tested for ribonuclease and deoxyribonuclease activity as summarized in Table 1.

and moving slightly toward the cathode. The major peak, itself, migrated only very slightly at the pH employed (citrate buffer, 0.05 M, pH 7.5) and is therefore essentially isoelectric at this pH value. A sample containing the small, more acidic components was removed at the end of the run and analyses for RNase and DNase activity are given in Table 1. This table also includes similar assays on the enzyme at various earlier stages of purification. The data show fairly convincingly that both activities are attributable to the same protein component, confirming the earlier impression of Alexander *et al.*[8]. The purified protein gives an entirely negative test for free SH groups by the method of Boyer[13], even at a level of 0.1 μmole per 2.5 ml. The absence of both cysteine residues and half-cystine residues is also shown by the results of amino acid analysis of hydrolysates of the protein after exposure to conditions of reduction and alkylation. Thus, no trace of S-carboxymethylcysteine was demonstrable nor was cystine itself detected. The amino acid analyses performed on the "reduced-alkylated" protein is summarized in Table 2. Since the protein is free of cysteine residues

Table 1. Relative activities of micrococcal nuclease against RNA and DNA at various levels of purification. RNase and DNase assays were performed on duplicate aliquots of each sample, which had been diluted with phosphate buffer (0.05 M, pH 6.1) to a suitable protein concentration.

	RNase activity $\Delta A_{260m\mu}$	DNase activity $\Delta A_{260m\mu}/15$	Activity ratio RNase/DNase
Dialyzed crude enzyme	0.123	0.032	3.84
After Ca++ precipitation	0.284	0.068	4.18
"Shoulder" from electrophoresis	0.090	0.030	3.00
Main sample from electrophoresis cell	0.314	0.081	3.87
Original sample for electrophoresis (see Fig. 3)	0.322	0.096	3.36

Table 2. Amino acid analyses of purified nuclease.

Amino acid	20 h hydrolysate		44 h hydrolysate		Average % of total μmoles	Assumed number of residues
	μmoles	% of total μmoles	μmoles	% of total μmoles		
Lysine	0.56	14.8	0.65	13.8	14.3	14
Histidine	0.08	2.1	0.08	1.7	1.9	2
Arginine	0.13	3.4	0.12	2.5	3.0	3
Aspartic acid	0.37	9.8	0.47	9.9	9.9	10
Threonine	0.25	6.6	0.30	6.3	6.5	7
Serine	0.12	3.2	0.14	3.0	3.1	3
Glutamic acid	0.47	12.4	0.60	12.7	12.6	13
Proline	0.17	4.5	0.23	4.9	4.7	5
Glycine	0.27	7.1	0.35	7.4	7.3	7
Alanine	0.35	9.2	0.45	9.5	9.4	9
Half Cystine	0		0		0	0
Valine	0.23	6.1	0.27	5.7	5.9	6
Methionine	0.08	2.1	0.06	1.3	(2.1)	(2)[a]
Isoleucine	0.12	3.2	0.17	3.6	3.4	4
Leucine	0.31	8.2	0.46	9.8	9.0	9
Tyrosine	0.19	5.0	0.25	5.3	5.2	5
Phenylalanine	0.09	2.4	0.11	2.3	2.4	2
Tryptophan						(1)[b]
Total μmoles	3.79		4.71			
Total residues						102[c]

[a] Loss of methionine is presumably due to inadequate evacuation of the hydrolysis tubes.
[b] Based on the presence of a single Ehrlich-positive peptide on "fingerprints".
[c] Assuming 2 moles of histidine/mole.

and disulfide bridges, the reduction-alkylation procedure is, in a sense, superfluous. The absence of disulfide bonds suggests that the calcium ions required for enzymatic activity may be involved in the three-dimensional stabilization of the polypeptide chain and experiments to test the role of calcium in the determination of tertiary structure are in progress. The enzyme contains approximately 100 amino acid residues/molecule on the basis of the amino acid analyses if one assumes the presence of two residues of histidine/molecule.

Peptide maps prepared on trypsin digests of the protein as described in the experimental section showed approximately 20 major peptide components and a few much less intensely staining ones. This number is roughly consistent with the analyses for lysine and arginine content. One of the peptides gave a positive test for tryptophan with the Ehrlich stain, and four were strongly positive for arginine, as indicated by spraying the maps with Sakaguchi reagent. Two other minor peptide components gave faint tests for arginine. The excellent separation

of peptides on the "fingerprints" and the absence of material at or near the origin indicate that the trypsin digest contains very few if any large components and should be ideally suited to sequence analysis.

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