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PURIFICATION OF AN ALKALINE NUCLEASE FROM *PHYSARUM POLYCEPHALUM*

JAAP H. WATERBORG and CHARLES M.A. KUYPER

Department of Chemical Cytology, Faculty of Science, University of Nijmegen, Toernooiveld, Nijmegen (The Netherlands)

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Summary

An alkaline nuclease was purified from microplasmodia of *Physarum polycephalum*. The nuclease, active on denatured DNA and RNA and free of contamination by other nucleolytic activities, appeared to be a zinc-metallo protein. The enzyme was only active under conditions, where Zn^{2+} were retained in the enzyme. Loss of zinc occurred by the chelating action of EDTA, EGTA or ampholines, by acid of highly alkaline pH conditions or by high ionic strength. The addition of $ZnCl_2$ to compensate losses, restored all activity, while all other divalent cations caused inhibition. The nuclease, with a molecular weight of 32 000, was stable at neutral pH at high temperatures with a half-life of 20 min at 80°C. It was inhibited by any salt of buffer concentration above the level of zero ionic strength and showed a special sensitivity towards phosphate ions.

The possible similarity of this enzyme to nuclease S1 from *Aspergillus oryzae* is pointed out.

Introduction

Several DNA-degrading enzyme activities are found in crude extracts of microplasmodia of the acellular slime mold *Physarum polycephalum* [1] and they show growth, cell cycle and differentiation-dependent variations in their activities [2].

Alkaline DNAases generally are thought to be involved in highly regulated processes, e.g. DNA replication in nuclei and mitochondria [3—9]. Elevated levels of alkaline DNA-degrading activities were found during the induction of sclerotization and in correlation with S-phase DNA replication in synchronous

surface plasmodial cultures of *P. polycephalum* [2]. This study describes the purification of an alkaline nuclease from microplasmodia after the induction to sclerotization.

Materials and Methods

Microplasmodia of *P. polycephalum* were cultured essentially according to Daniel and Baldwin [10] as modified by Werry [11] in 2-l Erlenmeijer flasks, each containing approx. 1 l semi-defined growth medium. At mid-logarithmic growth phase the microplasmodia were collected by centrifugation for 5 min at $200 \times g$, resuspended in non-nutrient salt medium [12] and growth was continued for 9 h to induce the onset of spherule formation. Enzyme purification from cultures later than 12 h after the transfer was not possible, because slime interfered with centrifugation and chromatography.

Nuclease assay. A mixture of 0.7 ml containing 0.1 ml enzyme solution in buffer A (10 mM Tris-HCl, 10 mM KCl, pH 7.0), 70 mM Tris-HCl, pH 8.0, and heat-denatured salmon sperm DNA (Sigma) at 3.6 mg/ml was incubated at 30°C for an appropriate time within the range of linear time and homogenate kinetics, which was normally 1 or 2 h with fractions diluted five or ten fold with buffer A. The incubation was stopped by chilling in melting ice and 0.05 ml bovine serum albumin (50 mg/ml in distilled water of bovine serum albumin fraction V, Sigma) and 0.5 ml of 2.5 N $HClO_4$ were added. The tubes were shaken vigorously, left standing in ice for 15 min and clarified by centrifugation for 10 min at $1500 \times g$ in the cold. The absorbance of the supernatants was measured at 260 nm with a Zeiss PM6 spectrophotometer and corrected for blank values.

One unit of enzyme activity was defined as the amount of enzyme which produced under the assay conditions during an incubation of 1 h at 30°C an increase in the absorbance at 260 nm of 1.0 in the acid supernatant.

In the assay for the detection of nuclease activity in the fractions during purification the concentration of DNA was reduced to 0.7 mg/ml or the DNA was replaced by yeast RNA (0.7 mg/ml, Schwarz), purified according to Shortman [13], or poly(A) (0.7 mg/ml, Sigma). $ZnCl_2$ (1.0 mM) was included in the nuclease assay of the isoelectric focusing fractions. A concentration of 210 mM Tris was used for the assay of the DEAE-cellulose fractions. In the assays with RNA or poly(A) 0.025 ml bovine serum albumin (50 mg/ml) was added together with 0.025 ml heat-denatured salmon sperm DNA (5 mg/ml, Sigma).

Nuclease assay on double-stranded DNA. 0.5 ml of 0.3 M Tris-HCl, pH 7.5, containing 1.4 mM $MnCl_2$, 0.1 ml of enzyme solution in buffer A and 0.1 ml native salmon sperm DNA (5 mg/ml in distilled water, Sigma) were incubated at 30°C and processed as described above.

Phosphodiesterase and phosphatase assays. 0.5 ml of 0.3 M Tris-HCl, pH 8.5, 0.1 ml enzyme solution in buffer A and 0.1 ml 16.7 mM bis-*p*-nitrophenylphosphate (Merck), *p*-nitrophenylthymidine 3′- or 5′-phosphate (Sigma) for the phosphodiesterase assays or *p*-nitrophenylphosphate (Merck) for the phosphatase assay were incubated at 30°C, stopped in ice and 0.5 ml 1 M Na_2CO_3

was added. The absorbance was measured at 410 nm and corrected for blank values.

The molecular weight of the nuclease was determined by chromatography in buffer A on Sephadex G-100 with standards of known molecular weight.

The protein content of fractions I—IV was determined according to Lowry et al. [14] and of fraction V according to McKnight [15] with minor modifications.

Nuclease fraction V with approx. 0.8 μg of protein and 100 units of enzyme activity was subjected to electrophoresis in a 7.5% discontinuous polyacrylamide gel system according to Davis [16]. Gels were sliced in 1 mm sections and the slices were eluted overnight in buffer A. The enzyme activity was measured on denatured DNA and yeast RNA as described. To study the protein composition of fraction V, 2 μg of protein were subjected to electrophoresis on the Davis gels and 8 μg on sodium dodecyl sulfate polyacrylamide gradient gels (6—18%) according to Laemmli [17]. Gels were stained with Coomassie brilliant blue R250 (0.05%, w/v) in methanol/acetic acid/distilled water (5 : 1 : 5), destained in acetic acid/methanol/distilled water (7 : 5 : 88) and the absorbance was measured at 590 nm.

The temperature stability of the nuclease was determined by incubation of the purified enzyme fraction V in buffer A at 0.25 μg of protein/ml at the given temperatures and for the described length of time. The remaining enzyme activity was measured on denatured DNA and poly(A) as described.

Results

Purification of the nuclease

All procedures were performed at 4°C, except when noted. Microplasmodia from twelve 1 l cultures in salt medium were collected by centrifugation for 5 min at 1000 × *g*, washed once with cold distilled water and twice with cold buffer A. To 1000 ml of plasmodial pellet 1.5 l of buffer A were added and Triton X-100 to a final concentration of 0.12% (w/v). The resuspended microplasmodia were sonicated in batches of 150 ml with a MSE 105 W sonifier for 5 min continuously at stage low-4 with cooling. The sonicate was called Fraction I. It was clarified by centrifugation in a JA-10 rotor in a Beckman Junior centrifuge at 10 000 rev./min for 1 h. The supernatant was called fraction II.

To the supernatant were added 1.5 kg of DE-52 DEAE-cellulose (Whatman) and the slurry was gently stirred for 1 h. The DEAE-cellulose was sucked dry on a Büchner filter, washed with an excess of buffer A and suspended in 6 l of buffer A. The thin slurry was left to settle by gravity in a column (8 × 60 cm) over a layer of fresh DEAE-cellulose. The column was washed at a flow rate of 6 ml/min with 3 l of buffer A and it was eluted with 9 l of a linear gradient from 10 to 410 mM KCl in 10 mM Tris-HCl, pH 7.0, and with 1.5 l of buffer A containing 0.4 M KCl. Fractions of 25 ml were collected. Nucleolytic activities were measured as described. The fractions with activity on denatured DNA and RNA (Fig. 1A) were pooled and concentrated 45 fold against dry polyethyleneglycol 6000 (Merck). The concentrate was dialyzed overnight against 2 l of buffer A and called fraction III.

Potassium phosphate buffer (pH 6.8) was added to fraction III to a concen-

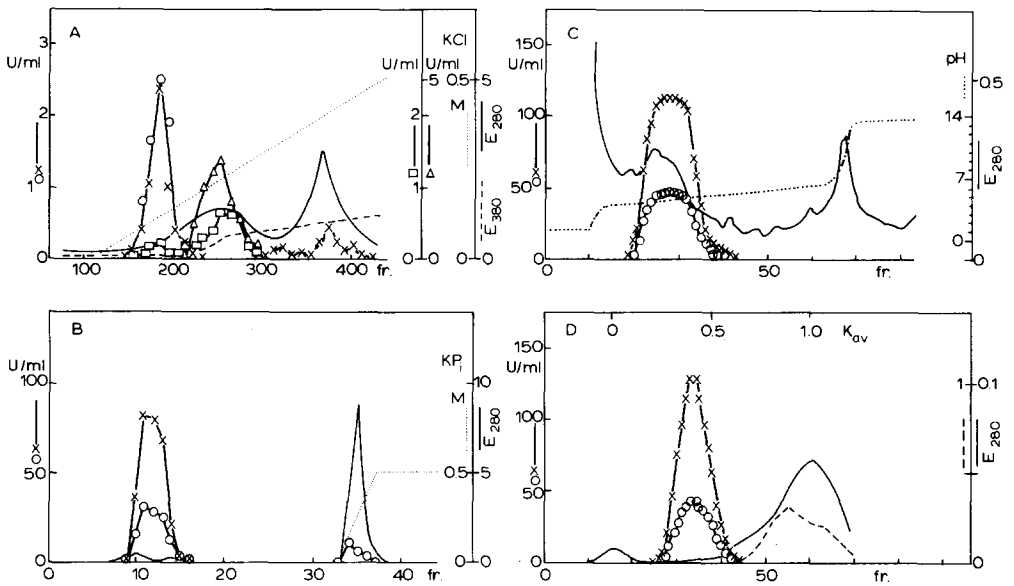

Fig. 1. Purification of the alkaline nuclease. All procedures are described in Materials and Methods and in Results. X———X, nuclease activity on denatured DNA; ○———○, nuclease activity on yeast RNA; ———, absorbance at 280 nm, fr, fraction. (A) Chromatography of fraction II over DEAE-cellulose, □———□, nucleolytic activity on native DNA; △———△, hydrolytic activity on bis-*p*-nitrophenylphosphate; · · · · · ·, concentration of KCl (M) during gradient elution; - - - - - -, absorbance at 380 nm, measurement of the yellow pigments. (B) Chromatography of fraction III over hydroxyapatite. · · · · · ·, concentration of potassium phosphate, pH 6.8 (M). (C) Isoelectric focusing of fraction IV. · · · · · ·, pH determined at 4°C. (D) Gel filtration of active isoelectric focusing fractions over Sephadex G-100. - - - - - -, absorbance at 240 nm, measurement of ampholines; K_{av}: partition coefficient, as defined [18].

tration of 1 mM. The sample was loaded on a column of hydroxyapatite (2.6 × 22 cm, DNA grade, Biogel), equilibrated with 1 mM potassium phosphate buffer, pH 6.8. The column was washed as a flow rate of 1 ml/min with 300 ml 1 mM potassium phosphate buffer, eluted with 60 ml of a linear gradient from 1 to 500 mM potassium phosphate and washed with 150 ml 500 mM potassium phosphate buffer. Fractions of 13 ml were collected. The fractions with activity on DNA and RNA (Fig. 1B) were pooled and called fraction IV.

Fraction IV was concentrated three fold against dry polyethyleneglycol 6000 and dialyzed overnight against 2.5 l of 2 mM Tris-HCl, pH 7.0. It was focused for 53 h at 600 V and 4°C in a 110 ml LKB isoelectric focusing column with ampholines (pH 4—6, 1%, w/v, LKB). The cathode was at the top with spacers devoid of enzyme between the enzyme-containing sucrose gradient and the two electrode solutions. Fractions 1.38 ml were collected at 0.7 ml/min, the pH of the fractions was measured at 4°C and nuclease activities on denatured DNA and yeast RNA were measured in assays with 1 mM $ZnCl_2$ to counter the chelating properties of the ampholines [19]. The active fractions at pH 4.65 (Fig. 1C) were pooled and $ZnCl_2$ in excess over the chelating ampholine concentration was added to a final concentration of 10 mM to quantitatively reactivate the nuclease activities. The pooled fraction was concentrated two fold against dry polyethyleneglycol 6000, chromatographed over a Sephadex G-100 column (2.6 × 34.5 cm), equilibrated with buffer A. The

column was eluted with buffer A at a flow rate of 1 ml/min and fractions of 3.2 ml were collected. The fractions with nuclease activity (Fig. 1D) were pooled and called fraction V.

The purification achieved was approximately 25 000 fold (Table I) and the recovery of nuclease activity in several preparations ranged from 6 to 10%. This low recovery did not all relate to the nuclease proper but, in part, to the removal of other enzymes with activity on single-stranded DNA or RNA.

Some steps in the procedure merit a short comment. The batch-wise treatment of fraction II with DEAE-cellulose was necessary before column chromatography, because the slime still remaining in the fraction clogged filterpaper and even nylon gauze with 0.1 mm pore diameter. For optimal and reproducible purification during hydroxyapatite chromatography the sample volume had to be less than 25% of the column volume, hence the concentration step. As the ampholines have chelating properties [19], they remove Zn^{2+} from the enzyme molecules. Exact quantification of the nuclease in the presence of ampholines, even after the addition of $ZnCl_2$, was not possible, therefore these had to be removed by sieving through Sephadex. As determined by chromatography on Sephadex G-100 the molecular weight of the nuclease was 32 000 ± 1000.

Although fraction V did not contain measurable activities of acid and alkaline phosphodiesterases, phosphatases, nucleases on double-stranded DNA or other nucleolytic activities, other than the one characterized, the nuclease was not pure. On polyacrylamide-SDS gels four major and some minor protein bands could be demonstrated in the range of 15 000—45 000 molecular weight. On 7.5% polyacrylamide gels two major and several minor protein bands could be detected. However, these bands did not coincide with enzyme activity (Fig. 2).

All futher characterizations of the enzyme activity were made with purified nuclease fraction V.

The enzyme was stable during all stages of the purification procedure. All of

TABLE I

PURIFICATION OF THE ALKALINE NUCLEASE

The purification procedure of the nuclease from microplasmodia of *P. polycephalum* after salt induction is described in Materials and Methods and in Results. The recovery of enzyme activity, measured by the nuclease assay on DNA, was corrected for the samples taken during the purification procedure.

Fraction	Volume (ml)	Protein (mg/ml)	Activity, yield			Specific activity (U/mg protein)	Purification
			U/ml	Units	%		
Fraction I, sonicate	2500	7.6	45	111 500	100	5.85	1.0
Fraction II, supernatant	2337	5.3	41	95 500	86	7.71	1.3
Fraction III, DEAE-cellulose	37	10.1	210	7 660	7	20.6	3.5
Fraction IV, hydroxyapatite	49	0.11	200	9 880	9	1 810	310
Fraction V, isoelectric focussing and gelfiltration	47	0.0013	180	8 540	8	140 000	24 000

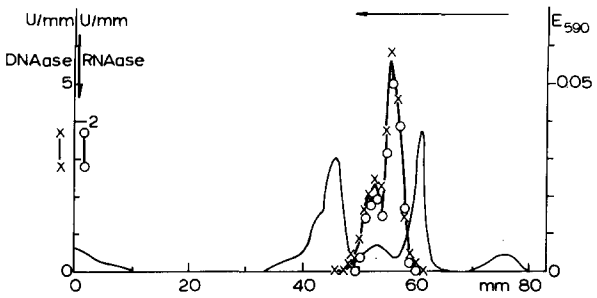

Fig. 2. Electrophoresis of nuclease fraction V. All procedures are described in Materials and Methods. The direction of electrophoresis was from right to left (←——) and the electrophoresis was stopped when bromophenol blue (←——) had reached the end of the gel. The nuclease activities on DNA (X——X) and RNA (○——○) were determined after elution. ——, absorbance at 590 nm, measurement of Coomassie brilliant blue.

the activity was retained when it was stored for several days in buffer A at 4°C and for 1 year in buffer A at −20°C even with repeated freezing and thawing and at protein concentration of 1 μg protein/ml or less. The nuclease was thermostable at neutral pH. The rates of inactivation of enzyme activity on denaturated DNA and on poly(A) were identical. The half-lives at 75, 78 and 82°C were 150, 40 and 10 min, respectively.

Characterization of the nuclease

The activity of the nuclease was inhibited by the chelators EDTA and EGTA and by nearly all divalent cations. A strong inhibition was found with $MnCl_2$ and moderate inhibitions with $CaCl_2$, $MgCl_2$ and $CuCl_2$ (Fig. 3). $ZnCl_2$ caused inhibition at concentrations over 1 mM, while a slight stimulation was found with Zn^{2+} below this concentration. The degree of stimulation was strongly dependent on the ionic strength and pH. Generally, less stimulation by low concentrations of Zn^{2+} was found at lower ionic strength and near the optimal pH of the enzyme activity.

To confirm the observation that Zn^{2+} were the only divalent cations essential for enzyme activity, fraction V was treated for 1 h at 20°C with EDTA at various concentrations and the remaining enzyme activity was measured without and with concentrations of several divalent cations in slight excess over the EDTA concentration. Reactivation of the enzyme to the original level of activity after the EDTA treatment was only possible with $ZnCl_2$ (Fig. 4). The other divalent cations had no effect.

The activity of the nuclease was strongly dependent on ionic strength. This characteristic was found with Tris buffer and with other salts like ammonium sulfate, NaCl and KCl (Fig. 5). Zero ionic strength conditions should be used to observe the highest possible enzyme activity, but this could not be realized, because without any buffer during the assay irregular kinetics were found due to pH drift. A moderate ionic strength of Tris buffer, 70 mM was used, was chosen to compromise between buffer capacity of the assay and inhibition by ionic strength.

Optimal enzyme activity with 70 mM Tris was found at pH 8, with 50% activity at pH 5.5 and 8.8. The Tris buffer could not be replaced by phosphate

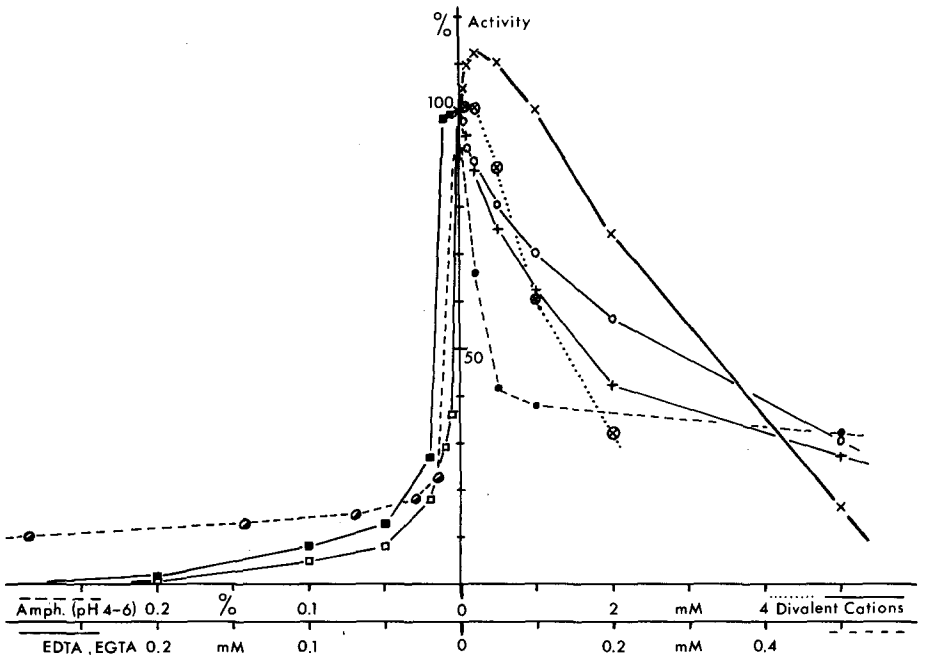

Fig. 3. Effect of divalent cations and chelating compounds. All tested compounds were dissolved in the buffer for the nuclease assay on denatured DNA (0.7 mg/ml). The range of concentrations was 0—0.5 mM for $MnCl_2$, 0—0.2 mM for EDTA and EGTA, 0—0.4% (w/v) for ampholines (pH 4—6) and 0—5 mM for $ZnCl_2$, $MgCl_2$ and $CaCl_2$. In all assays 210 mM Tris-HCl, pH 8.5, was used except in one series with $ZnCl_2$ (· · · · · ·) where 70 mM Tris-HCl, pH 8.0, was used. X———X, $ZnCl_2$; +———+, $MgCl_2$; ○———○, $CaCl_2$; ●- - - - - -●, $MnCl_2$; □———□, EDTA; ■———■, EGTA; ◕- - - - - -◕, ampholines; ⊗· · · · · ·⊗, $ZnCl_2$. All percentages are given relative to the enzyme activity without any additions.

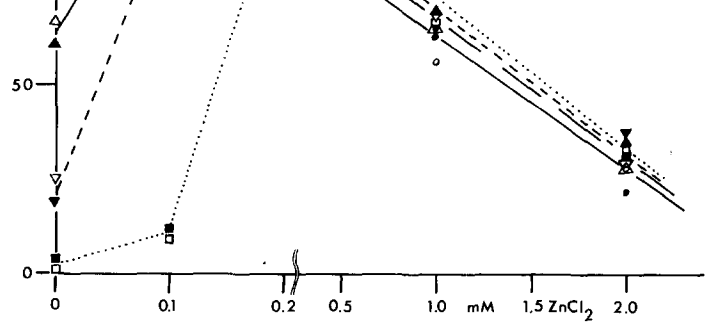

Fig. 4. EDTA inactivation and $ZnCl_2$ reactivation. EDTA was added to fraction V to a final concentration of 0 mM (○———●), 0.025 mM (▽· · · · · ·▼), 0.10 mM (△- - - - - -▲) and 1.00 mM (□· · · · · ·■). After 1 h at 20°C enzyme activities were measured on denatured DNA (open symbols; 0.7 mg/ml) and on yeast RNA (filled symbols; 0.7 mg/ml) in assays with 70 mM Tris-HCl, pH 8.0. The final concentrations of EDTA during the assays were 0.0, 3.6, 14.3 and 143 μM, respectively. All percentage are given relative to the enzyme activities without EDTA or $ZnCl_2$.

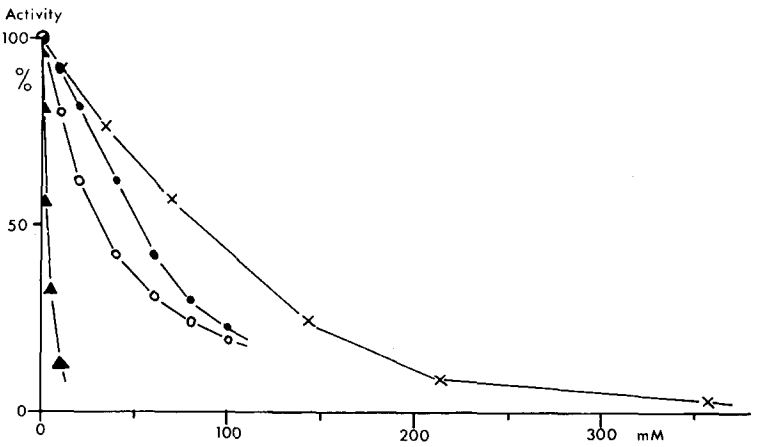

Fig. 5. Inhibition of nuclease activity. The effect of Tris-HCl, pH 8.0 (X———X), KCl assayed with 70 mM Tris-HCl, pH 8.0 (●———●), KCl assayed with 210 mM Tris-HCl, pH 8.5 (○———○) and potassium phosphate (pH 6.8) assayed with 210 mM Tris-HCl, pH 8.5 (▲———▲) was tested in the assay with denatured DNA (0.7 mg/ml). All percentages are given relative to the enzyme activity without additions.

buffer in the neutral pH range, because the phosphate ions gave a strong competitive inhibition even at very low concentrations. 50% enzyme activity was found at 2.5 mM phosphate (Fig. 5). The other buffer systems tested, sodium cacodylate-HCl and glycine/NaOH gave lower activities, 95 and 50%, respectively, relative to Tris buffer at identical pH values.

When tests were made to determine whether or not sulfhydryl groups were essential for enzyme activity, inhibition was found with 2-mercaptoethanol (50% activity at 1.25 mM) and with *p*-chloromercuribenzoate (50% activity at 0.5 mM). The inhibition by mercaptoethanol could be a direct effect, although binding of Zn^{2+} cannot be excluded. The major effect of *p*-chloromercuribenzoate in this system was the binding of Zn^{2+}, which resulted in a white insoluble complex and inactivation of the nuclease. The enzyme activity could be restored by the addition of excess $ZnCl_2$.

Discussion

The statement that the enzyme preparation fraction V contained a single nuclease, active on denatured DNA and RNA, was based on the following arguments.

1. Exact cochromatography of activity on both substrates was found with DEAE-cellulose, hydroxyapatite and Sephadex G-100 columns.

2. Identical patterns of activities on both substrates after electrophoresis in polyacrylamide gels or during isoelectric focusing.

3. Identical results for the activities on both substrates during the studies of temperature stability and of EDTA inactivation, $ZnCl_2$ reactivation of the enzyme.

According to these criteria sonicates from plasmodia in the logarithmic growth phase or during sclerotization contain the same enzyme, but the

amount was much higher under the latter conditions.

The final preparation contained no interfering enzymic activities, nucleases, RNAases, DNAases, phosphodi- and phosphomonoesterases and nucleotidases.

The enzyme showed an absolute requirement for Zn^{2+} and thus must be considered a metallo enzyme. The fact that not only chelating compounds like EDTA, EGTA or ampholines inactivated the enzyme, that also changes in pH or ionic strength could dissociate the Zn^{2+} and thereby inactivate the nuclease, makes it probable that the binding of the Zn^{2+} is electrostatic. From the cations especially Mn^{2+} was inhibitory and even Zn^{2+} itself in concentrations over 1 mM caused inhibition. Phosphate ions should be specially mentioned, because they caused very strong inhibition of the nuclease.

Preliminary experiments [19] indicate an endonucleolytic mode of action, a preference for bonds containing adenine and the formation of 5′-phosphorylated end products. The *Physarum* enzyme thus seems to have many characteristics in common with nuclease S1 from *Aspergillus*, also a zinc metalloenzyme [20], but it is maximally active at neutral or slightly alkaline pH.

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