J. Biochem. 88, 1715–1721 (1980)

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Nucleic Acids and Related Enzymes

Secretion of Four Alkaline DNases by Plasmodia of *Physarum polycephalum*

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Received for publication, July 7, 1980

In plasmodia of *Physarum polycephalum*, DNase activity with a preference for native DNA was found in a pattern of three or four isoenzymes. During growth a constant specific activity of approx. 0.3 unit of DNase activity per mg protein was found in the plasmodia, with a broad maximum during the G2-phase in the naturally synchronous flat cultures. Under conditions of starvation or sclerotization, DNase activity was secreted by the plasmodia in amounts which were up to ten times higher than the internal level of enzyme activity. Purification of the secreted DNase activity to high purity by three simple chromatographic steps showed that four different DNase isoenzymes existed which were identical with the intracellular ones. The relative abundances of the various isoenzyme forms inside and outside the plasmodia seemed to be slightly different. The possible functions of the DNase activities are discussed.

In plasmodia of the acellular slime mold *Physarum polycephalum*, alkaline DNase activity with a preference for native DNA is found in a pattern of at least three isoenzymes (*1*). In stationary cultures, secretion of alkaline DNase activity with a preference for native DNA was observed, but it was not clear whether this activity was identical with the intracellular DNase activity.

In this paper we present a comparison of the levels of intracellular and extracellular DNase activity under different physiological conditions, and of the pattern of isoenzymes. A procedure for the purification of the extracellular DNase isoenzymes is presented, which resulted in much purer preparations with higher recoveries than would be possible from plasmodia.

Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

MATERIALS AND METHODS

Microplasmodia of *Physarum polycephalum* (M3c) were cultured in the media and under the conditions described earlier (*2*) and surface plasmodial cultures were grown according to Guttes and Guttes (*3*) as modified by Werry (*4*).

Microplasmodia were sampled and crude extracts prepared as described elsewhere (*2*). Samples of media were taken and used as enzyme preparations without any treatment. Surface plasmodial cultures were taken between the second mitosis (M2) and 3 h after the third mitosis (M3+3h), after fusion of the microplasmodia. They were washed by dipping in cold distilled water and in buffer A (10 mM Tris-HCl, 10 mM KCl, pH 7.0), scraped off the filter paper and homogenized as described previously (*2*). The method

for changing media and for the addition of cycloheximide (10 μg/ml, Sigma) has been described (*2*).

Purification of the Extracellular DNase Activities—Microplasmodia were cultured in semi-defined growth medium. The plasmodia from 3 liters of culture in the near-stationary growth phase (approx. 100–125 ml of plasmodia per liter) were removed by centrifugation for 5 min at 200 $\times g$ in the cold. All procedures were performed at 4°C, except when otherwise noted. The growth medium was concentrated approx. four fold against dry polyethylene glycol 6000 (Merck) and dialyzed for 3 days against 2 changes of 5 liters of buffer A. A similar preparation was obtained when the growth medium was lyophilized until dry, taken up in ten to twenty percent of the original volume of buffer A and dialyzed against buffer A. The dialysate was called Fraction I.

Fraction I was mixed with 100 g of DEAE-cellulose (DE-52, Whatman), then the slurry was gently stirred for 1 h and left to settle by gravity in a column (6×16 cm) over a layer of 100 g of fresh DEAE-cellulose, equilibrated with buffer A. The column was washed by gravity flow at 3 ml/min with 0.65 liter of buffer A and eluted with 1.0 liter of a linear gradient from 0 to 0.4 M KCl in buffer A. Fractions of 25 ml were collected. DNase activities towards native and denatured DNA were determined as described below. The flow-through fractions, which contained DNase activity with a preference for native DNA, were pooled, concentrated 7-fold against dry polyethylene glycol 6000, and dialyzed for 2 days against 2 changes of 2.5 liters of 5 M urea, 2 mM Tris-HCl (pH 7.0); this fraction was called Fraction II.

Approximate half of Fraction II was focused for 90 h at 500 V and 4°C in a 110 ml LKB isoelectric focusing column with Ampholines pH 3.5 to 10 (1%, w/v, LKB) in a linear gradient from 5 to 45% (w/v) sucrose containing 5 M urea and with the cathode at the top. Fractions of 1.03 ml were collected at 0.7 ml/min. The pH of the fractions was measured at 4°C and DNase activities towards native and denatured DNA were measured as described below. Fractions with DNase activity towards native DNA were collected in such a way that each peak of enzymatic activity was pooled separately. The pooled activities from the acid (pH=5.7), neutral (pH=7.1), alkaline (pH=8.9), and highly alkaline (pH=9.4) regions of the column were called A, B, C, and D, respectively. They were each concentrated to 3 ml against dry polyethylene glycol 6000 and chromatographed over a column of Sephadex G-100 (2.6×68 cm) (Pharmacia), equilibrated with buffer B (5 M urea, 10 mM Tris-HCl, 10 mM KCl, pH 7.0). The flow rate was 0.2 ml/min and fractions of 4 ml were collected. The fractions with DNase activity towards native DNA were pooled, concentrated 10-fold and called Fraction IIIA, Fraction IIIB, Fraction IIIC, and Fraction IIID, respectively.

DNase Activity—DNase activity was measured essentially according to the nuclease assay described by Waterborg and Kuyper (*5*). The incubation mixture (0.7 ml) contained 0.1 ml of enzyme solution, 0.1 ml of native or heat-denatured herring sperm DNA (Boehringer), 1 mM $MnCl_2$, and 210 mM Tris-HCl (pH 7.6) for the determination in crude extracts and in growth medium or 35 mM Tris-HCl (pH 8.5) for the determination in fractions obtained during the purification procedure. The incubation at 30°C was stopped by cooling in ice. The incubation mixture was acidified with $HClO_4$ and the absorbance of the clarified supernatant (1.25 ml) at 260 nm was measured and corrected for blank values. One unit (U) of DNase activity was defined as described elsewhere (*1*). The activities of the zinc-metallo endonuclease enzyme towards DNA and RNA and of acid and alkaline phosphodiesterases and phosphatases towards bis-*p*-nitrophenylphosphate (Merck) and *p*-nitrophenylphosphate (Merck) were measured by the reported methods (*5*). Optimum conditions for enzyme activity, molecular weight (on Sephadex G-100) and mode of action were determined as described in (*1*) and protein, RNA, and DNA contents were measured as described in (*2*).

RESULTS

DNase Activity within Physarum Plasmodia—DNase activity with a preference for native, double-stranded DNA was found in plasmodia of *Physarum polycephalum*. The specific activity was constant during growth at approx. 0.2 to 0.3 unit of DNase activity per mg of plasmodial protein. In surface plasmodial cultures this value varied

slightly over the cell cycle and higher specific activities were consistently observed during G2-phase (Fig. 1A). A constant value was observed for microplasmodia growing in semi-defined growth medium, with a tendency to decrease somewhat during sclerotization (Fig. 1B). The constant level of specific activity under the growth conditions changed abruptly when microplasmodia were transferred from growth medium to a balanced salt medium used for the synchronous induction of sclerotization (Fig. 1C). The increase to approx. 200 percent relative to that under the growth conditions was very steep, and the activity remained high until sclerotia were formed, and was found both in the absence and in the presence of 10 μg/ml cycloheximide. The stepwise increase was also seen when microplasmodia were transferred to salt medium with glucose, which is a growth medium lacking protein. However, the level of specific activity of the DNase in plasmodia, cultured in growth medium, remained unchanged when plasmodia were transferred to fresh growth medium or to salt medium with protein, which is a growth medium lacking glucose.

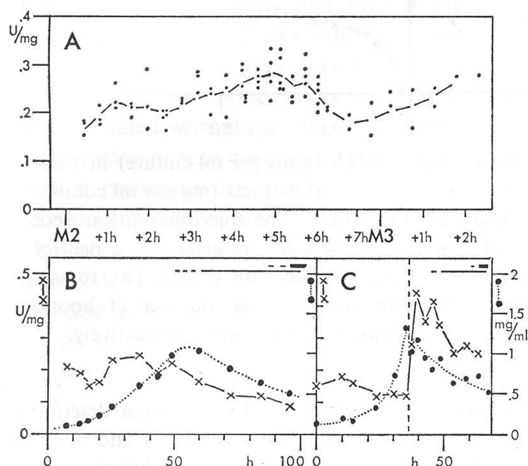

Fig. 1. Plasmodial DNase activity. A: DNase specific activity (units per mg protein) in surface plasmodial cultures. Each measurement (●) was obtained from a single plasmodium, and the average values (······) are indicated. B: DNase specific activity (×) in microplasmodia, growing in semi-defined growth medium. C: DNase specific activity (×) in microplasmodia in growth medium and after a transfer in salt medium. The time of transfer is indicated (······). DNase activity towards native DNA and protein content (mg per ml of culture, ●) were determined in the crude plasmodial extracts as described in "MATERIALS AND METHODS." The presence of slime and spherules is indicated at the top by the thin and thick lines, respectively.

Secretion of DNase Activity—The constant level of specific activity during growth signified that a continuous synthesis of DNase activity occurred in parallel with protein synthesis. The total amounts of both increased until the growth medium became exhausted and both decreased during the process of sclerotization. However, just before the highest amount of DNase activity was found within the plasmodia, DNase activity with a preference for native DNA started to appear in the growth medium, which up to that time had lacked all alkaline DNA-degrading enzyme activity (Fig. 2A). The rate of secretion appeared to be correlated in such a way with plasmodial density that approx. 21.5 mU of DNase activity was secreted per h and per mg of plasmodial protein in the range from 0.3 to 1.7 mg of protein per ml of culture. This rate of secretion remained nearly constant in semi-defined growth medium until sclerotia started to appear. Similar patterns of DNase secretion were observed when plasmodia were transferred from growth medium to salt medium with protein (Fig. 2B) or to fresh growth medium (results not shown). Secretion started some hours before the highest plasmodial protein and DNase contents were reached and stopped when sclerotia were formed. A transfer of microplasmodia to salt medium (Fig. 2C) or salt medium with glucose (Fig. 2D) resulted in an immediate secretion of DNase activity, even at plasmodial densities which did not give secretion in growth medium. However, a plasmodial density higher than 0.25 mg of protein per ml of culture was required for secretion to start upon a transfer to salt medium with or without glucose. Secretion of DNase activity stopped when sclerotia were formed. Because the time between the start of secretion and the formation of sclerotia was longest in growth medium, the highest amounts of secreted DNase activity were always found in this system.

Comparison of Isoenzyme Patterns—An attempt was made to compare DNase isoenzyme patterns in plasmodia and in growth medium without initial purification by ion exchange chromatography. This proved to be impossible.

Isoelectric focusing of growth medium gave clear peaks of the DNases with neutral and alkaline isoelectric points, but the peak of the DNase with an acid isoelectric point (pI=5.7) was obscured by the activity of plasmodial zinc-metallo endonuclease activity, which appeared in the growth medium from broken plasmodia. The amount of this activity with an isoelectric point of 4.6 was only two percent of the total plasmodial activity. Isoelectric focusing of crude plasmodial extracts was impossible because at still undetectable levels of DNase activity heavy precipitation of protein occurred, particularly in the acid range (pH 4.0 to 5.5), on the isoelectric focusing column.

The patterns of the plasmodial isoenzymes could be determined after extensive purification by DEAE-cellulose, hydroxyapatite and DEAE-cellulose chromatographies, the first three steps

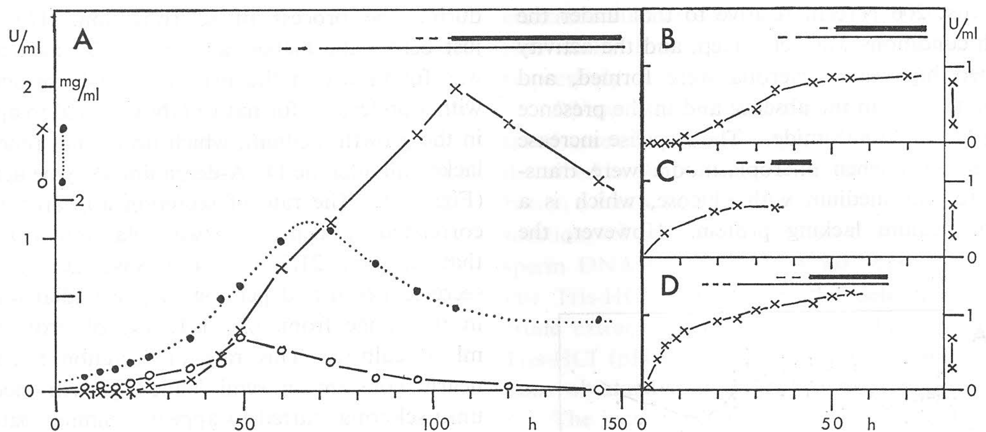

Fig. 2. Secretion of DNase activity. DNase activity towards native DNA (units per ml culture) in plasmodial extracts (○) and in the medium (×) and protein content in plasmodial extracts (mg per ml culture, ●) were determined as described in "MATERIALS AND METHODS." The microplasmodia were cultured in growth medium (A) for the indicated number of hours. Microplasmodia grown to a density of 0.4 to 0.5 mg of protein per ml of culture were transferred to salt medium with protein (B), to salt medium (C), and to salt medium with glucose (D), and cultured for the indicated number of hours. The presence of slime and spherules is indicated at the top by the thin and thick lines, respectively.

TABLE I. Relative abundance of DNase isoenzymes. The relative amounts of the DNases were determined in the isoelectric focusing fractions. Plasmodial extracts were focused after DEAE-cellulose (2×) and hydroxyapatite chromatography (*1*), while growth medium was focused after a single DEAE-cellulose chromatography as described in "MATERIALS AND METHODS." The activity of the minor form D was added to that of DNase C. Plasmodia were transferred to salt medium as described elsewhere (*2*). The results indicated by the asterisk (*) were obtained from a single culture.

DNase isolated from	Relative amount of DNase activity (%)		
	DNase A	DNase B	DNase C (+D)
1. Growth medium	10	83	7
2. Growth medium	8	85	7
3. Growth medium (*)	8	85	8
4. Growing plasmodia (*)	16	71	13
5. Plasmodia, 6 h after a transfer to salt medium	14	65	22
6. Plasmodia, 9 h after a transfer to salt medium	9	75	16

for the purification of the plasmodial enzymes (*1*). The isoenzyme pattern from growth medium could be determined after a single run on DEAE-cellulose had removed the contaminating nuclease activity. Table I gives a collection of data on the isoenzyme patterns obtained during the purification of the DNases from plasmodia and growth medium. Generally, DNase B with its neutral isoelectric point was always found as the most abundant form, both inside and outside the plasmodia. Its relative amount appeared to be somewhat higher outside, but the uncertainty as to whether isoenzyme-specific losses had occurred before the isoelectric focusing precluded definitive conclusions.

Purification of the Secreted DNase Isoenzymes—The secreted DNase isoenzymes were purified by a modification of the procedure developed for the purification of the intracellular enzymes (*1*). Some comments should be made on this. Chromatography of a plasmodial extract on DEAE-cellulose gave two peaks with activity towards native DNA. One peak was found in the flow-through fractions of the column. It was heavily contaminated with phosphatase, phosphodiesterase and nuclease activities and the DNase activity with a preference for native DNA could not be recovered from it before inactivation occurred. The second peak with DNase activity was eluted from DEAE-cellulose after the zinc-metallo endonuclease activity. Urea was added to this preparation to prevent loss of activity through complexation, as described previously (*1*). Chromatography of dialyzed growth medium was apparently carried out at protein concentrations low enough to prevent complexation and inactivation of DNase activity. All activity was recovered in the flow-through fractions of the column (Fig. 3). The contamination by nuclease, phosphodiesterase, and phosphatase activities, all plasmodial enzymes which appeared to a minor extent in the medium through breakage of plasmodia, was completely eliminated from the DNase preparation. Isoelectric focusing of this preparation gave four peaks with DNase activity at pH 5.7, 7.1, 8.9, and 9.4, and these were called DNases A, B, C, and D, respectively (Fig. 4). They all showed identical molecular weights of 17,000 during buffer exchange and Ampholine removal on Sephadex G-100. The three-step procedure, described in detail in "MATERIALS AND METHODS," gave DNase preparations which were free of contamination by nuclease, RNase, phosphodiesterase and phosphatase activities. The extent of purification, shown in Table II, was a minimum estimate because even after further concentration of the final preparations not enough protein was available for reliable determination. No Coomassie Brilliant Blue-stainable material was found when 1.6 ml of Fraction IIIB was concentrated by Amicon B15 ultrafiltration to 0.025 ml and electrophoresed on polyacrylamide-SDS gels (*5*). The purity obtained for the secreted DNases was at least 200 times higher than that of the plasmodial enzymes after a more extensive purification procedure.

The DNase isoenzymes purified from the

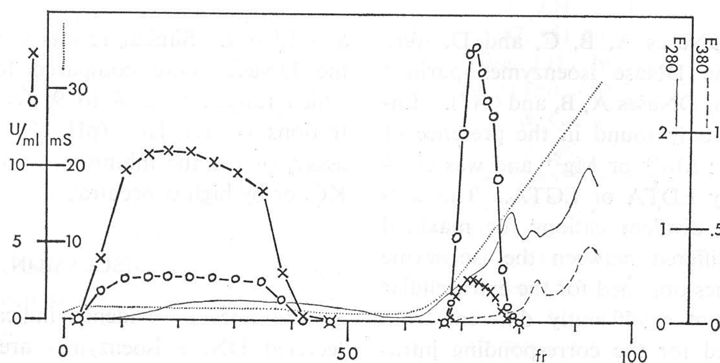

Fig. 3. DEAE-cellulose chromatography of DNase Fraction I. DNase activity (units per ml) towards native DNA (×) and denatured DNA (○), the absorbance at 280 nm (——), and 380 nm (– – –) and the conductivity (······) of the fractions were determined as described in "MATERIALS AND METHODS."

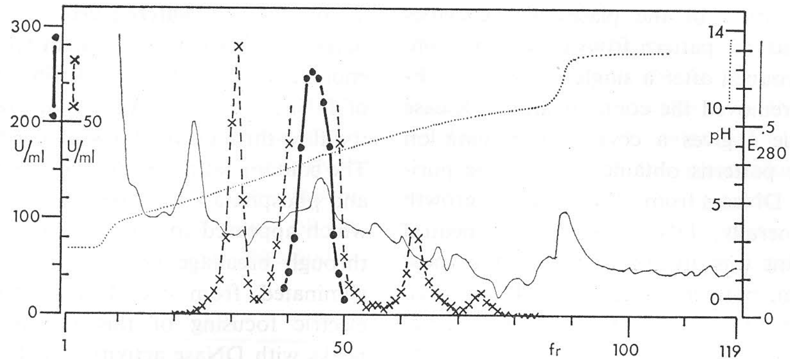

Fig. 4. Isoelectric focusing of DNase Fraction II. DNase activity towards native DNA (×: range 0 to 70 units per ml; ■: range 0 to 300 units per ml), the absorbance at 280 nm (——) and the pH (······) were determined as described in "MATERIALS AND METHODS."

TABLE II. Purification of four DNases from growth medium. DNase activity was purified from the medium of 3 liters of microplasmodial culture, as described in "MATERIALS AND METHODS." The protein contents of Fractions IIIA, IIIB, IIIC, and IIID were upper estimates obtained by the Lowry method. The ratio of the activities towards native DNA (dsDNA) and denatured DNA (ssDNA) was calculated as described previously (*1*). The recovery of enzyme activity was corrected for samples taken during the purification procedure.

Fraction		Volume (ml)	Protein (mg/ml)	Activity and yield (U/ml)	(units)	(%)	Specific activity (U/mg protein)	Purification X	Ratio dsDNA/ssDNA
I	Growth medium	821	1.30	13.4	10,996	100	10.3	1.0	0.82
II	DEAE-cellulose	133	0.112	35.25	4,700	43	315	30.5	3.7
IIIA	Isoelectric Focusing+ Sephadex G-100	6.2	0.005	36.8	227	2.1	7,214	700	5.5
IIIB		10.3	0.029	280.7	2,903	26.4	9,815	952	3.9
IIIC		11.7	0.010	17.8	208	1.9	1,799	175	2.4
IIID		7.0	0.009	8.9	63	0.6	998	97	2.3

growth medium, DNases A, B, C, and D, were compared with the DNase isoenzymes purified from the plasmodia, DNases A, B, and C (*1*). Enzyme activity was only found in the presence of the divalent cations Mn^{2+} or Mg^{2+} and was completely inhibited by EDTA or EGTA. The concentrations of the divalent cations for maximal enzyme activity differed between the isoenzyme forms, but the values obtained for the extracellular isoenzymes were not significantly different from the values obtained for the corresponding intracellular ones. The $MnCl_2$ optimal concentrations for extracellular DNases A, B, C, and D were 2.4, 1.4, 1.0, and 0.8 mM, respectively, while the highest activity with $MgCl_2$ was noted at 8, 4, 2.5, and 1.8 mM. Similar results were obtained when the DNases were compared for optimum pH, which ranged from 8 to 9, for optimal concentrations of Tris-HCl (pH 8.5) buffer during the assay, or for the inhibition of DNase activity by KCl or by high concentrations of urea (*1*).

DISCUSSION

Several lines of evidence indicate that the four secreted DNase isoenzymes are identical to the forms observed in and purified from the plasmodia of *Physarum polycephalum*. The peaks of activity of the DNases A, B, and C were found at identical pH values during isoelectric focusing when plas-

modial or secreted preparations were used. DNase D, which was detected as a minor form in the plasmodial preparations and was present in too small an amount for quantitative determination and enzyme characterization (*1*), could be isolated from the growth medium because the amount and the relative purity of DNase activity secreted were much higher than from the plasmodial material. The molecular weight of 17,000 was found for all DNase preparations on Sephadex G-100 in buffer B. The complexation of DNase activity to higher molecular weight forms in the absence of urea was not observed with the secreted activities, probably because the protein concentrations during the purification of the secreted activities were much lower. The enzymatic characteristics of the secreted DNases were identical with those of the plasmodial DNases as regards $MnCl_2$ and $MgCl_2$ activation, pH optimum, ionic strength, and urea inhibition, and mode of endonucleolytic DNA hydrolysis. The tendency of a lower preference for native DNA by the DNases with more alkaline isoelectric points also held for DNase D (Table II).

Because only small amounts of DNase activity were present in the plasmodia, because precipitation of protein prevented isoelectric focusing of crude plasmodial extracts, as was also noted by Hüttermann *et al.* (*6*), and because contamination by nuclease and phosphodiesterase activities obscured the peak of DNase A activity during isoelectric focusing of growth medium, no direct measurements of the relative amounts of the various isoenzymes could be made without prior purification, which could involve a preferential loss of one or more of the DNase forms. All four isoenzymes were found inside and outside the plasmodia, but their relative abundances seemed not to be identical (Table I). More work would be required to determine whether the differences observed are significant.

When plasmodia were transferred to salt medium with or without glucose, two phenomena were observed, which are probably related. First, the plasmodial total and specific activity of DNase increased approx. two fold in a step-wise manner and, secondly, secretion of DNase activity into the medium started. However, the mechanism by which the apparent DNase activity increases remains unclear. The absence of inhibition by cycloheximide of the plasmodial increase seems to rule out *de novo* enzyme synthesis. The activation of pre-existent DNase activity or the removal of DNase-inhibiting substances seems likely because the amount of DNase activity found in the growth medium increases to more than ten times the maximum amount found inside the plasmodia, even on the assumption that all activity towards native DNA in the crude extracts (*1*) can be ascribed to the enzyme studied.

The start of secretion of DNase activity appeared to be correlated with the exhaustion of the growth medium and with the process of sclerotization, which is induced by the condition of starvation (*2*). A function of the DNase activities for the digestion of food (intracelllular and under the condition of starvation also extracellular) seems improbable because digestive enzymes are mostly acid hydrolases of lysosomal origin which produce 3′-phosphorylated termini in digested nucleic acids, while the DNases studied are alkaline enzymes which give 5′-phosphorylated products. A regulatory function in the process of sclerotization might be indicated by the observations in bacteria and fungi that DNases, RNases, proteases, and other exoenzymes are secreted at the end of growth and that these enzymes appear to be essential for the formation of spores and sclerotia (*7*, *8*).

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