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ENDONUCLEASE ACTIVITY IN NUCLEI OF *PHYSARUM* *POLYCEPHALUM*

PARTIAL PURIFICATION AND CHARACTERIZATION

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Summary

An endonuclease, present in the microplasmodia of *Physarum polycephalum*, has been partially purified from isolated nuclei by DEAE-cellulose and Sephadex G-75 chromatography.

1. The endonuclease produced single-strand scissions in double-stranded DNA which resulted in the generation of 5'-phosphoryl and 3'-hydroxyl termini. No activity was observed with single-stranded DNA as substrate.

2. The pH optimum was approximately 8.5.

3. Divalent cations were essential for enzyme activity. $MnCl_2$ and $MgCl_2$ gave maximal activity. $CaCl_2$, $ZnCl_2$ or $CoCl_2$ did not activate the enzyme.

4. The endonuclease activity was highly sensitive to monovalent cations.

5. Endonuclease activity was found in two forms after gel filtration: an activity in a homogeneous peak with a molecular weight of approx. 20 000, and an activity that had a heterogeneous molecular weight and which was isolated in a complex with DNA.

A possible function of the endonuclease in DNA replication is discussed.

Introduction

Replication of DNA and its mode of regulation have been widely studied. Indications have been found that some DNAases are specific initiation factors for DNA replication [1–4] where they activate templates of DNA and chromatin for DNA polymerase activity. However, no specific DNAases have yet been characterized in and isolated from eucaryotes that function, in vivo, in the initiation of DNA replication.

Physarum polycephalum, an acellular slime mold, exhibits in its plasmodial

form a natural synchrony of its mitotic cycle and of its period of DNA replication [5–7] and is therefore an advantageous object for such studies.

Several DNA-degrading activities have been found in *P. polycephalum* [8–12]. They have not yet been fully characterized and their possible relationship to the various aspects of the DNA metabolism is unclear.

Results are presented of a partial purification and characterization of one of these DNA-degrading activities from nuclei. A part of these results has been published in a preliminary form [8]. Endonuclease activity was first observed as in total homogenates of microplasmidia [12]. Isolation of nuclei resulted in an 8-fold increase in specific activity of this endonuclease compared to that of total homogenates.

The type of endonuclease purified and studied could function as a DNAase which initiates the process of DNA replication [13].

Materials and Methods

Bacteriophage PM2, *Pseudomonas* BAL-31 and M13 DNA were kindly supplied by Dr. J.G.G. Schoenmakers. [³H]Thymidine was a New England Nuclear product, DEAE-cellulose DE-52 was from Whatman, Sephadex G-75 and dextran blue were from Pharmacia Fine Chemicals, bovine serum albumin fraction V and 2'-deoxyadenosine were from Sigma, polyethyleneglycol 6000 was from Merck, alkaline phosphatase, snake venom phosphodiesterase and calf spleen phosphodiesterase were from Worthington. All other chemicals were of reagent grade.

Microplasmidia of *P. polycephalum* [12] were cultured essentially according to Daniel and Baldwin [14] and Guttus and Guttus [15], as modified by Werry [16].

Nuclei were isolated from these microplasmidia essentially according to the nuclear isolation method with salt media as described by Polman [12].

Endonuclease activity was determined by measuring the conversion of superhelical substrate DNA into nicked forms with other sedimentation characteristics. The assay mixture (0.145 ml) contained 0.010 ml ³H-labelled PM2 DNA (0.2 µg), 0.100 ml enzyme in buffer A (5% glycerol (w/v), 14 mM 2-mercaptoethanol, 0.02% NaN₃ (w/v), 20 mM Tris · HCl, pH 8.4), 0.025 ml 0.5 M Tris · HCl (pH 8.5) and 0.010 ml 29 mM MnCl₂. The mixture was incubated for the appropriate time, dependent on the amount of enzyme present, at 30°C. The reaction was stopped by addition of 0.050 ml 0.1 M EDTA and cooling in melting ice. Analysis of the DNA was carried out on an alkaline sucrose gradient (4 ml, 5–20%) containing 0.4 M NaOH, 0.5 M NaCl, 1 mM EDTA [17]. Centrifugation was for 65 min at 60 000 rev./min at 4°C (Spinco rotor SW 60-Ti). Fractions of approx. 0.2 ml were collected, and the radioactivity was determined. In the incubation blanks the enzyme solution was replaced by buffer A. One unit of endonuclease activity was defined as the amount of enzyme which makes on average 1.0 scission per DNA molecule [18] during an incubation of 1 h with 0.2 µg PM2 DNA under the standard assay conditions. Precautions were taken to prevent activity of more than 2.5 breaks per molecule during the assay by adjustment of the time of incubation for endonuclease concentrations up to 200 units/ml.

In some experiments M13 DNA was used. The products were analysed on neutral sucrose gradients (4 ml, 5–20%) containing 1 M NaCl, 3 mM EDTA, 50 mM Tris · HCl, pH 7.6. Centrifugation was for 3 h at 55 000 rev./min at 4°C (Spinco rotor SW 56-Ti).

Radioactive PM2 phage was prepared with 2 μ Ci/ml [3 H]thymidine (20 Ci/mmol) and 50 μ g/ml 2'-deoxyadenosine [19,20]. The labelled DNA was extracted from polyethyleneglycol 6000-purified phage [21] and it always contained a nicked fraction of 20–30% [17,22].

Molecular weights of enzyme fractions were determined by chromatography on Sephadex G-75 with standards of known molecular weight.

Analysis of the termini which result from the nicking of DNA by the endonuclease was done essentially according to Suzuki and Sakaguchi [23] and Lavin et al. [24].

Protein and DNA contents were determined according to Lowry et al. [25] and Burton [26], respectively, after extensive dialysis of the samples against 10 mM Tris · HCl (pH 8.5) to remove the strongly interfering thiol compounds dithiothreitol or 2-mercaptoethanol [27]. DNA content of enzyme fractions was also determined by radioactivity. Microplasmidia were labelled for 26 h at 0.65 μ Ci/ml [3 H]thymidine (20 Ci/mmol). The specific activity of the DNA (in cpm per mg DNA) was determined in the isolated nuclei.

Results

Endonuclease activity in nuclei of P. polycephalum

Endonuclease activity was observed in total homogenates and isolated nuclei of *P. polycephalum* microplasmidia [8,12].

Incubation of circular superhelical M13 or PM2 DNA with the endonuclease showed that the superhelical DNA (RF I) was converted to relaxed circular DNA (RF II) containing one or more single-strand nicks (Fig. 1). No linear double-stranded DNA (RF III) was found. Incubation of a similar amount of circular single-stranded M13 DNA with the endonuclease gave no indication of cleavage.

For our enzyme preparations a linear relationship was found between the time of incubation and the number of single-strand scissions made in PM2 DNA until about 2.5 nicks per DNA molecule were made. The number of scissions also increased linearly with the amount of enzyme between 0.25 and 200 units/ml.

Enzyme purification

The endonuclease was purified from isolated nuclei of *Physarum* microplasmidia (Fraction 1, see Materials and Methods).

The endonuclease activity was solubilized from the nuclei by sonication in the presence of $(\text{NH}_4)_2\text{SO}_4$. To the nuclei, isolated from a 500 ml culture of *Physarum* microplasmidia, 2 ml of buffer B (1 M sucrose/5 mM MgCl_2 /1 mM dithiothreitol/10 mM Tris · HCl, pH 8.5) were added and 2 M $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 0.3 M [28]. The suspension of nuclei was sonicated in ice for 6×10 s with a MSE 150 W sonifier (stage: low-4) with cooling breaks of 20 s. After addition of 2 ml of buffer C (25% glycerol (w/v), 5 mM MgCl_2 ,

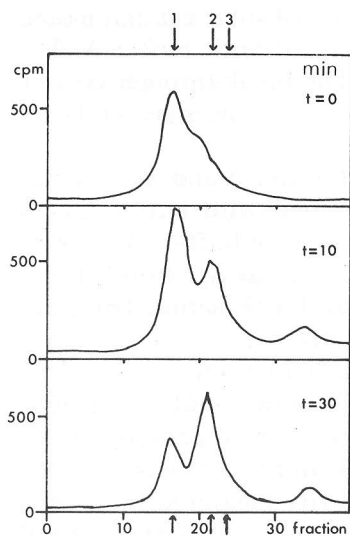


Fig. 1. Endonuclease activity with M13 RF I DNA substrate. The ^{32}P -labelled M13 DNA was incubated for the indicated number of minutes and analyzed on neutral sucrose gradients as described in Materials and Methods. Sedimentation was from right to left. Incubations of M13 DNA without enzyme showed no change in sedimentation pattern with increasing time of incubation. 1, M13 RF I DNA; 2, M13 RF II DNA; 3, M13 RF III DNA.

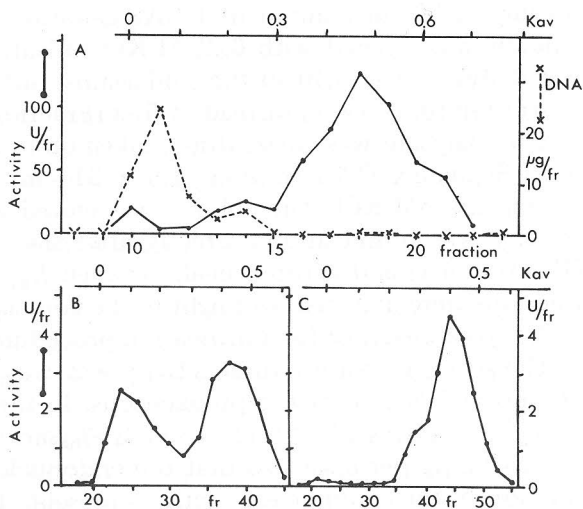


Fig. 2. Gel filtration of the endonuclease on Sephadex G-75. An 80 ml column of Sephadex G-75 (1.6 \times 31 cm) was used. Fractions were collected and endonuclease activity was determined by the standard assay. \bullet — \bullet , endonuclease activity, \times — \times , *Physarum* DNA determined by radioactivity as described in Materials and Methods. (A) Chromatography of freeze-dried Fraction 3 with buffer A containing 25 mM KCl. (B) Rechromatography of freeze-dried Fraction 4A with buffer A containing 1 M KCl. (C) Rechromatography of freeze-dried, sonicated Fraction 4A with buffer A containing 25 mM KCl. Sonication was done with a 150 W MSE sonifier (stage: high-4) for 10 \times 10 s. The void volume ($K_{av} = 0$) and total volume ($K_{av} = 1$) of the columns were determined by the elution of dextran blue and glycerol.

0.1 mM EDTA, 1 mM dithiothreitol, 50 mM Tris \cdot HCl, pH 8.5) the sonicated solution was centrifuged for 30 min at 30 000 \times g. The supernatant was dialyzed overnight in the cold against buffer A (Fraction 2).

TABLE I
PURIFICATION OF THE ENDONUCLEASE

The results of a typical purification of the endonuclease. Endonuclease was purified from the nuclei of 500 ml logarithmic culture of microplasmidia of *P. polycephalum*. Endonuclease activity, protein and DNA content were determined as described in Materials and Methods.

Fraction	Activity		Protein (mg/ml)	Specific activity (Units/mg protein)	Yield (%)	Purifi- cation	DNA ($\mu\text{g}/\text{ml}$)
	units/ml	units					
1. Nuclei	9.3	70.0	13.75	0.68	100	1.0	1045
2. Sonicate	3.0	43.6	3.32	0.91	62	1.3	248
3. DE-52 wash	6.7	440	0.32	20.8	629	30.6	16
4A. G-75 ($K_{av} = 0-0.3$)	9.4	960	0.097	97	1371	143	3.7
4B. G-75 ($K_{av} = 0.3-0.6$)	28.0	2824	0.028	1001	4034	1473	0.23

The dialysate was brought to 0.25 M KCl by addition of solid salt and passed through a 50 ml column of DEAE-cellulose equilibrated with buffer A. The column was washed with 0.25 M KCl in buffer A. The break-through volume was dialyzed overnight in the cold against buffer A but at a lower glycerol concentration (0.5% (w/v) instead of 5%) (Fraction 3).

The dialysate was freeze-dried, taken up in 1 ml of buffer A and then loaded on a Sephadex G-75 column (1.6 × 31 cm) equilibrated with buffer A containing 25 mM KCl. The column was eluted with this same buffer. A peak of activity was found at and directly after the void volume (at K_{av} 0–0.3 [29]) (Fraction 4A) and another peak between K_{av} 0.3 and 0.6 (Fraction 4B). Both fractions were dialyzed overnight in the cold against buffer A.

A typical result of the purification procedure is shown in Table I.

The strong increase in total activity was caused by the removal of endogenous *Physarum* DNA from the preparations. In the first nuclear preparation (Fraction 1) there was a 500-fold excess of *Physarum* DNA over the radioactive PM2 DNA (0.2 µg per assay) so that the endonuclease activity measured was only a fraction of the endonuclease activity present. The ammonium sulphate effected a removal of *Physarum* DNA of about 50% as an insoluble nucleoprotein complex [28]. More than 90% of the DNA from Fraction 2 was retained on the DEAE column which resulted in an apparent 10-fold increase in total activity. The complete removal of *Physarum* DNA from Fraction 3 by the gel filtration resulted again in Fraction 4B in an apparent 5–10-fold increase in total activity. It is, therefore, impossible to estimate enzyme recoveries at the several steps of the purification procedure.

The most active fraction (Fraction 4B) did not contain detectable amounts of DNA (less than 0.3% of the total *Physarum* DNA) and no further increases in total activity were found after preincubation experiments or after rechromatography over Sephadex G-75.

In Fraction 4A endonuclease activity was found together with approx. 3–5% of the total *Physarum* DNA, both on Sephadex G-200 and G-75 gels (Fig. 2A). Rechromatography experiments indicated that the endonuclease activity and the *Physarum* DNA were associated in a stable complex. This complex could only be split by strong sonication or high salt treatment. Rechromatography over Sephadex G-75 after these treatments showed that the endonuclease eluted as a homogeneous peak at the same K_{av} values as Fraction 4B (Figs. 2B and 2C) and had similar enzyme characteristics. Complete dissociation of endonuclease and DNA could not be achieved without major loss of enzyme activity.

Interference during the assay with the Sephadex G-75 fractions between $K_{av} = 0$ and $K_{av} = 0.3$ (Fractions 10–14, Fraction 4A, Fig. 2A) made exact measurements of the endonuclease activity in Fraction 4A impossible. During the alkaline sucrose gradient centrifugation part of the labelled PM2 DNA substrate was pelleted. This phenomenon, which is probably due to proteins present in Fraction 4A, was absent in all other elution fractions. It was also absent in the fractions with endonuclease activity between $K_{av} = 0.3$ and $K_{av} = 0.6$ after Fraction 4A was dissociated and rechromatographed on Sephadex G-75. Probably proteins that co-chromatographed with but were different from the endonuclease were responsible for this effect.

Characterization of the endonuclease

The sodium azide present in the preparations from Fraction 2 onward to prevent bacterial contamination, had a slight inhibitory effect on the purified enzyme of approx. 20%.

As 10 mM *p*-chloromercuribenzoate inhibited the endonuclease for over 60%, sulfhydryl protecting substances, dithiothreitol or 2-mercaptoethanol, were added during the purification and the standard assay of the endonuclease activity.

The purified enzyme (Fraction 4B) was active over a broad pH range, with optimum activity at pH 8.5 (Fig. 3).

Divalent cations were indispensable for activity of the purified enzyme. Maximal activity was found for fraction 4B with 2 mM MnCl_2 ; MgCl_2 at all concentrations tested, showed maximally only approx. 25% of this activity (Fig. 4). The endonuclease activity of Fraction 4A was also dependent on divalent cations. Here MgCl_2 at higher concentrations was the more activating ion (Fig. 5). The exact concentration of MgCl_2 for maximal activity varied from preparation to preparation, possibly dependent on the concentration of *Physarum* DNA still present, which could bind Mg^{2+} . Generally, higher activities were found if the endonuclease was assayed at a high concentration of MgCl_2 (10 or 15 mM) than under the standard assay conditions with 2 mM MnCl_2 . A similar activation by divalent cations as shown in Fig. 4 for Fraction 4B was found for the endonuclease activity ($K_{av} = 0.3-0.6$) when Fraction 4A was dissociated and rechromatographed as shown in Fig. 2.

For both fractions (4A and 4B) there was no replacement of the activation by Mn^{2+} or Mg^{2+} by CaCl_2 , ZnCl_2 or CoCl_2 . However, in the presence of Mg^{2+} or Mn^{2+} , Ca^{2+} stimulated the activity (Table II).

Monovalent cations were strongly inhibitory. 100 mM KCl or NaCl gave inhibitions of 70 and 80%, respectively. Inhibition of the endonuclease by 50 and 95% was found at KCl concentrations of 50 and 350 mM, respectively.

The enzyme in Fraction 4B was stable for at least 4 months at -20°C in buffer A. Repeated freezing and thawing had no effect. The temperature stability of the enzyme in Fraction 4B was tested by preincubations in buffer A at 55 and 65°C . The half-lives of the endonuclease activity were 16 and 12 min, respectively.

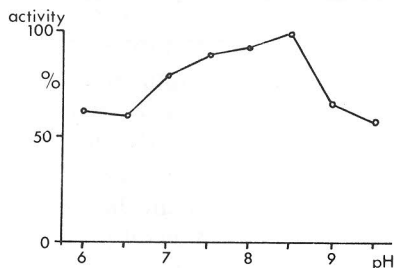


Fig. 3. pH optimum of Fraction 4B. In the standard assay the following buffer systems were used at 0.5 M: at pH 6.0 and 6.5 sodium cacodylate · HCl; at pH 7.0 sodium cacodylate · HCl/Tris · HCl; at pH 7.5, 8.0, 8.5 and 9.0 Tris · HCl; at pH 9.5 glycine · NaOH/Tris · HCl.

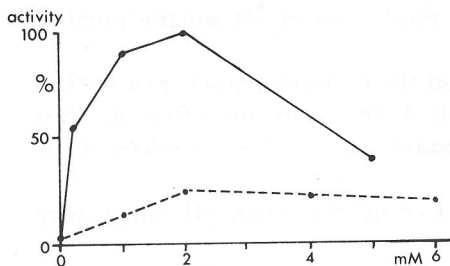


Fig. 4. Divalent cation optimum of the endonuclease, Fraction 4B. In the standard assay with PM2 DNA substrate the $MnCl_2$ solution was varied in concentration or replaced by $MgCl_2$ solutions. ●—●, $MnCl_2$; ●- - -●, $MgCl_2$.

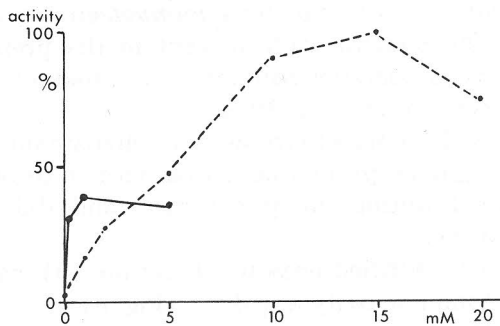


Fig. 5. Divalent cation optimum of the endonuclease, Fraction 4A. In the standard assay with PM2 DNA substrate the $MnCl_2$ solution was varied in concentration or replaced by $MgCl_2$ solutions. ●—●, $MnCl_2$; ●- - -●, $MgCl_2$.

The molecular weight of the endonuclease activity in Fraction 4B was estimated by rechromatography on Sephadex G-75 with a number of standards, and was found to be 20 500.

The termini produced by scissions of the endonuclease in PM2 DNA were analyzed (Table III). The snake venom phosphodiesterase, which acts on 3'-hydroxyl termini, gave acid-soluble products from endonuclease-treated PM2 DNA both with and without alkaline phosphatase pretreatment. The calf spleen phosphodiesterase, which acts on 5'-hydroxyl termini, gave maximal acid-soluble products only after the phosphatase treatment. Therefore we conclude that scission by the endonuclease produced 3'-hydroxyl and 5'-phosphoryl groups.

TABLE II

ENDONUCLEASE ACTIVITY OF FRACTION 4B WITH DIFFERENT DIVALENT CATIONS

Endonuclease activity of Fraction 4B with different divalent cations. In the standard assay with PM2 DNA substrate $CaCl_2$ was added to the $MnCl_2$ solution or the $MnCl_2$ solution was replaced by solutions with $CaCl_2$ and/or $MgCl_2$. The values given between parentheses were calculated relatively to the activity found with 5 mM $MgCl_2$.

Standard assay with	Activity (%)
2 mM $MnCl_2$	100
2 mM $MnCl_2$ + 0.5 mM $CaCl_2$	114
2 mM $MnCl_2$ + 2 mM $CaCl_2$	103
5 mM $MgCl_2$	46 (100)
5 mM $MgCl_2$ + 0.5 mM $CaCl_2$	68 (147)
5 mM $MgCl_2$ + 2 mM $CaCl_2$	49 (107)
0.5 mM $CaCl_2$	0
2 mM $CaCl_2$	0

TABLE III

ANALYSIS OF THE TERMINI PRODUCED BY THE ENDONUCLEASE IN PM2 DNA

Analysis of the termini produced by the endonuclease in PM2 DNA. PM2 DNA substrate was digested with Fraction 4B under the standard assay conditions for 26 h, which resulted in about 50 scissions per molecule. The reaction was stopped by heating (8 min, 100°C) in order to inactivate the endonuclease and to denature the PM2 DNA. Alkaline phosphatase (40 munits in 10 μ l 500 mM Tris · HCl, pH 8.5) was added. The incubation (45 min, 37°C) was stopped by heating (8 min, 100°C). Snake venom phosphodiesterase (6 munits in 10 μ l 10 mM MgCl₂/10 mM Tris · HCl, pH 8.5) or calf spleen phosphodiesterase (0.5 munits in 60 μ l 0.01% Tween 80/35 mM EDTA/650 mM sodium acetate, pH 5.0) was added. The incubation was for 22 h at 37°C. Carrier DNA and an equal volume of 1 M HCl in 70% ethanol were added and the acid-soluble radioactivity was determined. Appropriate blanks were treated in parallel.

	Alkaline phosphatase	Snake venom phosphodiesterase		Calf spleen phosphodiesterase	
		—	+	—	+
		cpm %	cpm %	cpm %	cpm %
Untreated PM2 DNA	—	0 0	0 0	0 0	36 10
	+	0 0	45 8	95 26	155 43
Endonuclease-treated PM2 DNA	—	221 37	661 111	170 47	209 58
	+	264 44	594 100	191 53	359 100

Discussion

An endonuclease has been purified from nuclei of *Physarum* microplasmodia. This enzyme acted on double-stranded DNA and produced single-strand scissions. In the most purified fraction (4B) practically all *Physarum* DNA was removed from the enzyme preparation, which made possible an exact determination of the total enzymatic activity. In all other, less purified preparations, the total endonuclease activity was underestimated because the *Physarum* DNA competed with the labelled PM2 DNA substrate. A similar underestimation was found with other nucleases, like the apurinic endonuclease from *Phaseolus* chromatin [30] or the endoRNAase from HeLa cell nucleoli [31], and similar increases in total enzymatic activity were noted upon the removal of nucleic acids from the preparations.

Elution of the endonuclease activity from Sephadex gels in free form (Fraction 4B) together with a heterogeneous form in a complex with DNA (Fraction 4A) has been found before [32]. This complexed activity could be dissociated, although part of the endonuclease activity was lost. Divalent cation activation data gave supporting evidence that the complexed activity (Fraction 4A) contained the same endonuclease as was found in free form (Fraction 4B). It was maximally active at high concentrations MgCl₂ while both the free activity (Fraction 4B) and the dissociated activity showed similar activation curves with maximal activity at 2 mM MnCl₂.

Associations between endonucleases and DNA have been found in the initiation complexes for DNA replication of plasmids [33–35] and ϕ X 174 [1]. It is unknown to which sequences of *Physarum* DNA the endonuclease is complexed in the several fractions and whether this DNA is intact or nicked, double-stranded or single-stranded. Further study of the complex could give

indications about the possible function, *in vivo*, although the possibility cannot be excluded that the complex is formed during the solubilization procedure of the endonuclease.

DNAases that are implicated as initiation factors for the replication of DNA all have similar enzymatic characteristics, which are also observed in the purified endonuclease from *Physarum*. They are found in nuclei, are dependent on activation by divalent cations, are active at slightly alkaline pH values on double-stranded DNA and make single-strand nicks that are terminated by 5'-phosphoryl and 3'-hydroxyl groups [2-4,36-38]. The *Physarum* endonuclease could be related to a specific group of these DNAases, the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent endonucleases [2,37-39] because CaCl_2 stimulation was found in the presence of the essential Mg^{2+} or Mn^{2+} .

Study of the template activation, *in vitro*, and the variations of activity in the cell cycle, *in vivo*, could further correlate the purified endonuclease from *Physarum* nuclei to the process of the initiation of DNA replication.

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